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Riboflavinyl Glucoside: a New Derivative of Riboflavin

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(*Received* 22 *May* 1951)

Homogenates of rat liver and aqueous extracts of acetone powders prepared from rat liver were shown to produce an unknown flavin, when incubated with riboflavin; it was reported that the reaction was enzymic (Whitby, 1950). The preparation and characterization of this new compound, which has been identified as a glucoside of riboflavin, is described here.

EXPERIMENTAL

Methods

Detection of enzymic activity. The production of riboflavinyl glucoside is detected by paper chromatography (Consden, Gordon & Martin, 1944; Whitby, 1950). Samples are prepared for chromatography by Crammer's (1948) method. The compound is not found in control experiments (samples taken at the beginning of an incubation, or after incubating riboflavin with liver preparations previously heated for 5 min. at 100°). The amount formed in any experiment is determined semi-quantitatively by visual comparison of the intensity of fluorescence with that of known amounts of riboflavin run simultaneously on the chromatograms; or, quantitatively, by separating a band of the mixed flavins and measuring the absorption at 450 mμ. in a Beckman spectrophotometer after elution of the glucoside with water, suitable correction being made for impurities in the filter paper.

Criteria of purity. (1) Separation from all contaminants which could be detected on chromatograms by their fluorescence in ultraviolet light was determined in several solvents (see Table 1). (2) An absorption spectrum of constant shape (aqueous solution). (3) A constant empirical mol.wt., as determined by the light absorption at 450 mμ. (Absorption coefficient, $\beta = 2{\cdot}80 \times 10^7$ cm.^{2}/mol.) (4) A sharp m.p.; this was determined with the Kofler micro-m.p. apparatus (A. H. Thomas Co., Philadelphia, U.S.A.) and is a corrected value.

Preparation of the compound

An acetone powder of rat liver was made as soon after death as possible: twenty livers were blended with 400 ml. of acetone, at 0°, for 1 min. in a Waring blender; the suspension was filtered rapidly on a Büchner funnel, and the solid pressed between filter papers. The solid was broken up, blended again with 400 ml. of acetone at 0°, filtered, pressed between filter papers, dried in a vacuum desiccator over H_2SO_4 and NaOH, and ground to a powder in a coffee mill. In all, 120 rats were killed and 180 g. of powder obtained; 5 g. batches were extracted by grinding in a mortar with 250 ml. of distilled water. This preparation was as active a source of the enzyme as the equivalent amount of liver homogenate, prepared according to Hunter's (1949) conditions.

The enzymic solution was incubated in lots of 1·5 l. (30 g. powder) with 300 mg. of riboflavin at 37°, in darkness for 6 hr. The solution was then deproteinized and desalted by a technique which is a large-scale development of Crammer's (1948) method: 100 ml. of trichloroacetic acid (50% w/w) were added, and the suspension cooled rapidly to 0°; 800 g. of $(NH_4)_2SO_4$ were added and the precipitate centrifuged off; the yellow solution was shaken with 300 ml. of aqueous phenol in a separating funnel, when the flavins were concentrated in the phenol.

Six batches were prepared in this way and the phenol concentrates combined (vol. 2 l., including washings). A sample of this phenolic solution was analysed by chromatography, and the main flavin components (riboflavin and the new flavin) were estimated by their light absorption at 450 mμ. (Table 2, *A*).

Table 1. *R_F values of flavins*

(Whatman no. 1 paper.)

Solvent systems*	*n*-Butanol, 4; acetic acid, 1; water, 5†	*n*-Butanol, 2; *n*-propanol, 2; water, 1	*n*-Butanol, 77; formic acid, 10; water, 13	Water, saturated with *iso*amyl alcohol‡
	R_F			
Riboflavin	0·30	0·20	0·13	0·4
Flavin mononucleotide	0·10	0·04	0·02	0·85
Flavin-adenine dinucleotide	0·03	0	0	0·9
Riboflavinyl glucoside	0·20	0·10	0·04	0·5
Lumiflavin	0·40	0·30	0·28	0·25
Lumichrome	0·70	0·55	0·50	0·1

* Composition of solvents is given in terms of volume ratios.
† Upper layer used as mobile phase.
‡ Ascending chromatogram.

Table 2. *Stages in the isolation of riboflavinyl glucoside*

	Yield of glucoside (mg.)	Ratios of light absorption at different wavelengths: 375 mμ. / 450 mμ.	275 mμ. / 450 mμ.	260 mμ. / 450 mμ.	Empirical mol.wt.
(*A*) Phenol concentrate	280 (+1·6 g. riboflavin)	—	—	—	—
(*B*) After shaking phenol concentrate with water and ether	230 (+300 mg. riboflavin)	0·86	—	7·0	—
(*C*) Effluent from first series of paper columns	200 (traces of riboflavin, etc.)	0·86	2·65	3·38	—
(*D*) Effluent from second series of paper columns	160 (chromatographically pure)	0·85 (4)	2·07	2·37	—
(*E*) Crystalline material	120	0·85 (2)	2·03	2·25	579
(*F*) Recrystallization from water	80	0·85 (2)	2·03	2·25	564
(*G*) Recrystallization from 80% (v/v) aqueous ethanol	60	0·85 (2)	2·03	2·25	540
(*H*) Second recrystallization from 80% (v/v) aqueous ethanol	40	0·85 (2)	2·03	2·25	540

Purification of the compound

It was not practicable to separate the compounds present in the phenol concentrate directly on paper columns, because of the small capacity of these columns for flavins, and the preponderance of riboflavin was reduced by shaking the phenol concentrate with 200 ml. water and 2 l. ether. Most of the new flavin was extracted into the aqueous phase, but relatively little of the riboflavin (240 mg. glucoside; 500 mg. riboflavin). The flavins were re-extracted into 200 ml. aqueous phenol; this phenol solution was shaken with 20 ml. water and 200 ml. ether, and the composition of the aqueous layer is shown in Table 2, Stage *B*. The limit of this technique had now been reached, and the flavin was next purified by chromatography.

Paper columns were made with Brown's 'Solka Floc' (B.W. Grade, 200 mesh). The paper was washed thoroughly before use by boiling for 0·5 hr. under reflux with water, then with 5% (w/v) acetic acid, water, 7% (w/v) K_2CO_3 to remove peptide material (Hird, 1950), water, *n*-butanol, water and finally acetone; it was spread on trays to dry. The columns were made in long glass tubes (5 cm. diam.), which were drawn out at one end; a plug of glass wool was put in the tapered end to support the column. The paper was poured on as a slurry in acetone, in accordance with the method of Campbell, Work & Mellanby (1951). No pressure was applied to the columns at any stage (cf. Peterson & Reineke, 1949). The rate of flow was controlled by a needle valve.

The separation of the new flavin from riboflavin on a paper column was accompanied by so great a broadening of the individual bands that it was necessary to select a solvent in which the R_F value of the new flavin was greater than the R_F value of riboflavin. This limited the choice of solvent to salt solutions and to water; it was advisable to add *iso*amyl alcohol to improve the separations effected by these solvents (Carter, 1950), but the separations were not as sharply defined as those obtained with solvents which were dependent upon partition mechanisms for their action. Water, saturated with *iso*amyl alcohol, was the solvent chosen for the first series of columns.

After packing, each column was washed with 50% (v/v) aqueous ethanol, and then with water. A washing with 5% glycine was included at this stage, to avoid the decomposition of flavins which otherwise occurred on the column. The glycine was washed out with water, and the columns equilibrated with the solvent. The capacity was very small owing to the low solubility of the flavins; a column built from 600 g. of paper could separate 15 mg. of the new flavin from 20 mg. of riboflavin. The rate of flow was adjusted to 25 ml./hr., and the separation followed by means of an ultraviolet lamp. Although this phase was tedious, riboflavin was almost completely removed from the mixture.

The effluent from the columns was extracted with ether (to recover the *iso*amyl alcohol), and the flavin solution evaporated to dryness in the dark, at 45°, under reduced pressure, and in an atmosphere of nitrogen. Despite these precautions, there was some decomposition with the formation of lumichrome. Besides this impurity, small amounts of riboflavin and of flavin mononucleotide were present. Although this series of columns had greatly decreased the content of impurities which absorbed light below 300 mμ. (Table 2, *C*), it was still possible to remove impurities which absorbed light in this region. These impurities could not be identified by the technique of Markham & Smith (1949), because the flavin trailed seriously on the chromatograms at concentrations which were too low to allow of the detection of purines, pyrimidines, etc., by this method, even if the total impurity had been due to a single compound.

The solvent system, *n*-butanol, 77; formic acid, 10; water, 13, was used on a second batch of columns, as trial chromatograms had shown that this solvent effected the greatest reduction in the light absorption below 300 mμ., relative to the 450 mμ. absorption. The solvent was theoretically capable of resolving all the fluorescent components of the mixture and the R_F value of the new flavin in this solvent differed from the R_F values of a wide range of derivatives of nucleic acids (Markham & Smith, 1949; Smith & Markham, 1950).

The columns were made as previously described; after glycine had been removed by washing with water, the columns were washed with 50% (v/v) aqueous ethanol, then with 85% (v/v) aqueous ethanol, before being finally equilibrated with the solvent (the components were distilled to remove their non-volatile residue). The flavin mixture was

dissolved in the minimum amount of solvent, and the band of the new flavin was collected in fractions; the solvent was removed by distillation under reduced pressure (in darkness, at 45°, and in an atmosphere of nitrogen), and all fractions which had a ratio of light absorption at 260 mμ.: 450 mμ. greater than 2·40 were repurified on a later column (Table 2, *D*). The overall yield up to this stage was about 55%. Paper chromatography showed that the compound had been freed from all fluorescent contaminants, and that little further alteration in the absorption spectrum could be effected.

production of riboflavin; the reaction is completed within 2·5 hr. in N-hydrochloric acid at 100°.

Absorption spectrum (Fig. 1). The molecular absorption coefficient at 450 mμ. is the same as the value reported for riboflavin by Singer & Kearney (1950); this value, which is about 8% higher than the value reported by Warburg & Christian (1938), has been confirmed with a potentiometrically pure sample of riboflavin. This sample was also found

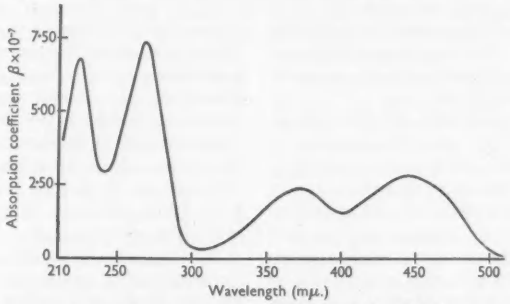


Fig. 1. Absorption spectrum of riboflavinyl glucoside in water.

Crystallization. The flavin was crystallized by evaporating an aqueous solution (about 20 mg./ml.) under reduced pressure at room temperature, a current of N_2 being made to impinge upon the surface of the solution. About 0·5 mg. of dry crystalline material was dissolved in 25 ml. water; the density of the solution at 450 mμ. was measured and the empirical mol.wt. calculated (Table 2, *E*). The compound was recrystallized as shown in Table 2, and 10 mg. of the final preparation (stage *H*) were recrystallized from acetic acid; the empirical mol.wt. of the specimen was 554, but the solution smelt slightly of acetic acid and attempts to remove it were prevented by shortage of material. No preparation was obtained with an empirical mol.wt. lower than 540±3 and this material (Table 2, stage *H*) was used for determining the m.p. and elementary composition.

The new compound had m.p. 247–248° (the crystals turned light brown in colour at 246°). Only carbon, hydrogen, nitrogen (and oxygen) could be detected. (Found: C, 51·1; H, 5·6; N, 10·3. $C_{23}H_{30}O_{11}N_4$ requires C, 51·3; H, 5·6; N, 10·4%.)

Molecular weight. An estimate of the mol.wt. was obtained with a micro-modification of Rast's method (Reilly & Rae, 1948). The compound had only slight solubility in camphor, so the molecular weight obtained by this method was approximate. The value was 500±50. $C_{23}H_{30}O_{11}N_4$ requires mol.wt. 538·5.

Characterization of the new compound

Stability. An aqueous solution is stable at 100°, but N-sodium hydroxide at 100° rapidly destroys the flavin by attacking the *iso*alloxazine nucleus. Lumiflavin is formed under the alkaline conditions described by Warburg & Christian (1938). The compound is hydrolysed by strong acids, with the pure by chromatography. It had m.p. 280–282°. (Found C, 54·2; H, 5·3; N, 15·0. Calc. for $C_{17}H_{20}O_6N_4$: C, 54·3; H, 5·3; N, 14·9%.)

The positions of the maxima and minima in the absorption spectrum of the new flavin coincide with the values for riboflavin; at the same molar concentration, their absolute heights coincide, except in the region 440–310 mμ., where the spectrum of the new flavin is consistently lower, the difference being very small at the extremes of this range, but gradually increasing to a difference of 3% at 400 mμ. and of 2% at 375 mμ.; the rest of the spectrum coincides with the spectrum of riboflavin within the limits of experimental error. The reasons for the difference between the spectrum of the new flavin and the spectrum of riboflavin are unknown.

The identity of the attached group was first investigated by chromatography after hydrolysis. Riboflavin interfered on chromatograms because of its intense yellow colour, but this interference was eliminated by dissolving the compound in ammonia (sp.gr. 0·880) and refluxing at 100° for 30 min.; riboflavin was treated similarly as a control. Ammonia was removed *in vacuo*, and the solid residue was dissolved in 2N-hydrochloric acid and heated for 2 hr. at 100°, after which hydrochloric acid was removed *in vacuo*. Samples of the hydrolysate were run on chromatograms, and glucose was identified as a product of hydrolysis with four different solvent systems, two of which were mixtures recommended by Jermyn & Isherwood (1949). Glucose was not released by refluxing with ammonia, as glycosidic links are stable to alkali.

Table 3. *Manometric estimation of* D-*glucose released from riboflavinyl glucoside by hydrolysis with acid*

(Manometer contents; main compartment: 2 ml. N-HCl; 0·25 ml. NaOH (40 g./100 ml. water); 0·5 ml. M-phosphate buffer (pH 6·0); 25 mg. ethanol; side bulb: 0·2 ml. notatin (400 μg./ml.); 0·1 ml. catalase.)

Amount hydrolysed (mg.)	Uptake of oxygen (μl.)	Glucose (mg.)			Recovery (% theory)
		Observed	Corrected*	Theoretical	
3·348	128	1·03	1·07	1·12	95·5
2·701	104·5	0·84	0·87	0·90	97

* Correction applied for loss of glucose during hydrolysis.

Estimation of D-*glucose.* The presence of D-glucose in the new derivative of riboflavin was proved, and the amount estimated manometrically, by means of notatin (Keilin & Hartree, 1948).

The compound was hydrolysed at 100° with N-hydrochloric acid for 2·5 hr., in a Warburg manometer cup fitted with an air condenser; the completeness of the hydrolysis was checked by chromatography. The acid was neutralized and D-glucose estimated manometrically. Control experiments were performed with known amounts of glucose and riboflavin; even with these mild conditions of hydrolysis there was a significant loss of D-glucose, amounting to 4%. The results of this experiment are shown in Table 3.

The point of attachment of D-*glucose to riboflavin; reaction with sodium metaperiodate.* The uptake of periodate was estimated by the method of Forrest & Todd (1950). The progress of the reaction is shown in Fig. 2; the uptake was 4 mol. periodate/mol. flavin.

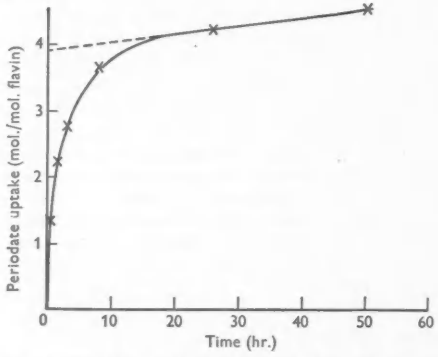
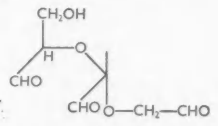


Fig. 2. Reaction of riboflavinyl glucoside with sodium metaperiodate.

Formaldehyde was estimated among the reaction products by the method of Boyd & Logan (1942); the quantity was less than one-quarter of the amount which would have been formed if the molecule had contained the structure CH_2OH—$CHOH$—.

Glucose is therefore attached either to the 4′ or 5′ position of riboflavin. The uptake of periodate shows that the formula must be either 6-(4′D-riboflavinyl)-D-glucopyranose or 5′D-riboflavin-D-glucopyranoside, i.e. riboflavinyl glucoside. The former alternative is excluded because glucose would be linked to riboflavin by an ether linkage, which would be stable to hydrolysis; the latter is held to be the correct alternative, because the glycosidic link is stable to hydrolysis with alkali and labile to hydrolysis with acid, and these are the observed stabilities of the linkage.

In riboflavinyl glucoside, 2 molecules of periodate are taken up by the glucose residue and 2 molecules by the riboflavin residue. The small amount of formaldehyde detected among the products of the reaction of the flavin with periodate is thought to arise from overoxidation of the end product (I):



(I)

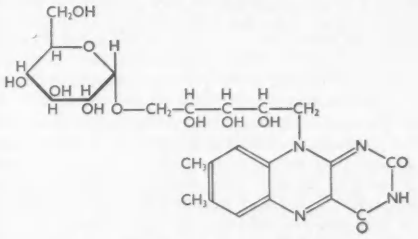
Overoxidation by periodate has been observed with similar compounds (Lindstedt, 1945; Halsall, Hirst & Jones, 1947), and a secondary breakdown of this type would produce, from this hypothetical end product, a compound capable of reacting further with periodate to form formaldehyde. The presence of formaldehyde among the products of the periodate titration could then be explained, as well as the slow reaction with periodate which occurs after the initial, comparatively fast, uptake of 4 mol./mol. flavin has been completed.

The proposed formula, riboflavinyl glucoside, was confirmed by the negative result given by the compound with the dinitrosalicylic acid reagent of Sumner & Sisler (1944), which indicated that the reducing group of glucose was involved in the linkage to riboflavin.

The configuration of the glucosidic link. This has not been definitely established, but in view of the following evidence it is suggested that the compound is an α-glucoside: (1) Sweet almond emulsin had no effect upon riboflavinyl glucoside. The very active β-glucosidase in this preparation hydrolyses

a wide range of β-glucosides (Pigman, 1944). (2) A preparation of α-glucosidase from dried baker's yeast (Krieble, Skau & Lovering, 1927) caused a slow breakdown of riboflavinyl glucoside to riboflavin; this preparation also hydrolysed maltose and α-methylglucoside, but was not shown to be entirely devoid of β-glucosidase activity. Different yeasts, grown under different conditions, produce α-glucosidases which between them can hydrolyse a number of α-glucosides (Gottschalk, 1950), but no single α-glucosidase has been prepared which is free from β-glucosidase and which has a wide range of action on α-glucosides. (3) The enzyme which produces riboflavinyl glucoside could be precipitated from the aqueous extract of the acetone powder of rat livers by half-saturation with ammonium sulphate; the precipitate dissolved in water, but the solution had no enzymic activity, unless an extract made by boiling a rat liver with water was added to the solution. Activity could also be restored by an aqueous solution of maltose or of glycogen, but not of glucose; α-glucose-1-phosphate inhibited the reactivation (preliminary experiments indicate that this inhibition is competitive). It is concluded that the enzyme catalyses a transglucosidation from maltose or glycogen to riboflavin. No transglycosidation has been reported which involves inversion of the configuration at the glycosidic link of the transferred carbohydrate residue (Bell, 1947).

The proof of the configuration of the glucosidic link may have to await chemical synthesis of the α- and β-glucosides. Meanwhile, the formula (II) is proposed as the probable structure of this new derivative of riboflavin.



II. 5′D-Riboflavin-D-glucopyranoside (riboflavinyl glucoside).

DISCUSSION

Riboflavinyl glucoside is a new derivative of riboflavin; this derivative of the vitamin was not observed by Crammer (1948) in any of the tissues which he investigated, but the reason may have been limitations in chromatographic technique. Riboflavin has been detected by the author in a concentrated extract of rat liver, although Crammer (1948) only detected riboflavin in preparations from spleen; further concentration of tissue extracts may reveal the natural occurrence of this new flavin. Bessey, Lowry & Love (1949) reported the presence of small amounts of riboflavin in various tissues, but their fluorimetric procedure was not designed to distinguish between riboflavin and its glucoside. Riboflavinyl glucoside has not been detected in urine; it would therefore appear to be different from a urinary flavin, the existence of which was postulated by Koschara (1935).

No function has so far been ascribed to the compound. It may undergo further metabolism and become incorporated into new nucleotides; flavin-X is a flavin dinucleotide of unknown structure (Sanadi & Huennuekens, 1950) and it is conceivable that riboflavinyl glucoside forms part of the molecule of flavin-X.

It should be possible to assess the significance of riboflavinyl glucoside when quantitative studies of the enzymic reaction have been completed, and the enzymic specificity determined. The combination of riboflavin with glucose may be one of a range of transglycosidations catalysed by the enzyme; the specific role of riboflavin will be tested by replacing riboflavin with some of the synthetic *iso*alloxazine derivatives as acceptors of glucose in the enzymic reaction.

SUMMARY

1. A new derivative of riboflavin, prepared by incubation of riboflavin with an enzyme obtained from rat liver, is described.

2. The elementary composition of the substance is $C_{23}H_{30}O_{11}N_4$; it has been identified as 5′D-riboflavin-D-glucopyranoside (riboflavinyl glucoside).

3. It is suggested that the glucosidic linkage has the α-configuration.

4. Preliminary investigations of the enzymic reaction indicate that the enzyme catalyses a transglycosidation of D-glucose from maltose or glycogen to riboflavin.

The author is indebted to Dr P. N. Campbell for useful discussions about paper columns, and to Messrs Johnsen Jörgensen and Wettre Ltd., 26 Farringdon Street, London, E.C. 4, for their gift of Solka Floc; to Prof. A. Albert for a sample of potentiometrically pure riboflavin; to Dr H. S. Forrest and Mr S. H. Christie for performing the periodate titration; and to Dr A. R. Trim for providing notatin and catalase. The analyses were carried out by Drs Weiler and Strauss, Oxford.

It is a pleasure to acknowledge the advice and encouragement of Dr M. Dixon, F.R.S., and the financial assistance of a grant from the Medical Research Council.

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Note on the Synthesis of the Acetic Acid Analogue of Thyroxine

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The acetic acid analogue of thyroxine has been prepared in order to determine its biological importance; biological experiments will be described in a later paper.

EXPERIMENTAL

Melting points are not corrected for emergent stem.

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*benzyl alcohol*. 3:5-Diiodo-4-(4′-methoxyphenoxy)benzaldehyde (Harington & Barger, 1927) (6·5 g.) was boiled under reflux with benzene (15 ml.) and 26 ml. of a solution of aluminium *iso*propoxide in *iso*propanol made from 5·5 g. Al and 200 ml. *iso*propanol. When a test portion of the reaction mixture no longer gave a precipitate with 2:4-dinitrophenylhydrazine, the product was extracted with dilute HCl, and the crystalline residue was collected and crystallized from 70% (v/v) ethanol. The yield was almost quantitative; after a further crystallization from benzene, the compound had m.p. 111–112°. (Found: I, 52·4. $C_{14}H_{12}O_3I_2$ requires I, 52·7%.)

3:5-*Diiodo*-4-(4′-*methoxyphenoxy*)*benzyl chloride*. The above alcohol (3·6 g.) was dissolved in $CHCl_3$ (20 ml.) and cooled in ice-salt, and powdered PCl_5 (1·83 g.) was added in small portions. At the end of the addition, the solution was kept at room temperature for 1 hr., washed with water, dilute $NaHCO_3$ solution, and water again, and dried over $CaCl_2$; on concentrating the solution, the product separated and was collected and crystallized from ethanol. The yield was 3·6 g. (96%). After distillation of a small amount *in vacuo* and crystallization from acetic acid, the compound had m.p. 96·5–97°. (Found: I, 50·7. $C_{14}H_{11}O_2ClI_2$ requires I, 50·8%.)

3:5-*Diiodo*-4-(4′*methoxyphenoxy*)*benzyl cyanide*. The preceding compound (2·25 g.) was dissolved in ethanol (13·5 ml.) containing a trace of KI and boiled under reflux for 4 hr. with KCN (0·45 g.) in water (0·9 ml.); the solution was concentrated to a low volume under diminished pressure and diluted with water, when the product separated. The yield was 2·025 g. (91%). After crystallization from acetic acid, the compound had m.p. 140–142°. (Found: N, 2·7; I, 52·3. $C_{15}H_{11}O_2NI_2$ requires N, 2·9; I, 51·8%.)

3:5-*Diiodo*-4-(4′-*hydroxyphenoxy*)*phenylacetic acid*. The nitrile (1·525 g.) was boiled under reflux for 1 hr. with red P (1 g.) and a mixture of acetic acid (10 ml.) and hydriodic acid, sp.gr. 1·7 (12 ml.). After removal of P the solution was concentrated to dryness, treated with water containing a little bisulphite and concentrated again. The product was dissolved in hot 0·1 N-Na_2CO_3 solution, filtered and acidified at the boiling point with dilute HCl. On cooling, 1·235 g. (80%) of the acid separated; after crystallization from 50% (v/v) acetic acid it had m.p. 214–216·5°. (Found: I, 50·8. $C_{14}H_{10}O_4I_2$ requires I, 51·2%. 9·0 mg. of the acid required 1·74 ml. of 0·0106 N-NaOH for neutralization to phenolphthalein, whence mol.wt. = 490; calc. mol.wt., 496.)

3:5-*Diiodo*-4-(3′:5′-*diiodo*-4′-*hydroxyphenoxy*)*phenylacetic acid*. The iodination offered some difficulty at first, as the conditions used for the preparation of thyroxine from diiodothyronine gave only gummy products which could not be purified; eventually success was achieved by the following method: the diiodo acid (25 mg.) was dissolved in methanol

(1 ml.), and concentrated NH_3 (1 ml.) and cooled in an ice bath; 0·2 ml. of N-I_2 solution was added very slowly with shaking. When the I_2 had all reacted, the solution was diluted with a few ml. of water and most of the NH_3 was removed under diminished pressure at 0°; the solution was then treated with 1 drop of 10% sodium metabisulphite ($Na_2S_2O_5$) solution and acidified at 40° with 3·5 N-HCl. The precipitate which separated was almost entirely crystalline. The yield was 34 mg. (91%). After crystallization from 2 ml. 50% (v/v) methanol, 22 mg. of the compound separated in long needles which had m.p. 219–220° (decomp.). (Found: I, 67·4. $C_{14}H_8O_4I_4$ requires I, 67·9%.)

SUMMARY

The synthesis of 3:5-diiodo-4-(3′:5′-diiodo-4′-hydroxyphenoxy)phenylacetic acid, the acetic acid analogue of thyroxine, is described.

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Some Observations on the Determination of Serum Protein Levels in Cattle

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The copper sulphate specific gravity method of Phillips, Van Slyke, Hamilton, Dole, Emerson & Archibald (1943) has found wide application as a rapid procedure for the determination of serum proteins in man and in certain other species. It has not been adapted to cattle. A recent investigation under field conditions entailed the determination of serum proteins in a large number of zebu cattle and the opportunity was taken to calibrate the specific gravity method against the tyrosine method of Greenberg (1929) which had been adopted as a convenient routine procedure. The Greenberg method has since been standardized on British cattle by comparing the results with those obtained using the usual macro-Kjeldahl technique and, as an independent check, tyrosine was also determined by the spectrophotometric procedure of Goodwin & Morton (1946).

METHODS

Macro-Kjeldahl procedure. Digestions were carried out in triplicate on 2 ml. serum, using the digestion mixture described by Chibnall, Rees & Williams (1943) and heating for at least 8 hr. after the solutions cleared. $Na_2S_2O_3$ (5% w/v) was added to the 40% (w/v) NaOH used to make the digest alkaline (Kantiengar, 1951). Non-protein nitrogen (N.P.N.) was determined by the method of Folin & Wu (1919) with nesslerization. After subtraction of N.P.N., nitrogen was converted to protein using the factor 6·25.

Tyrosine method (Greenberg, 1929). Readings were made in the Hilger-Spekker photoelectric absorptiometer, 30 min. after addition of the Folin-Ciocalteu reagent, against a 200 mg./l. tyrosine solution as standard.

Spectrophotometric determination of tyrosine (Goodwin & Morton, 1946). A 1 in 100 dilution of serum in 0·1 N-NaOH was found to give suitable E values. Readings were made in the Unicam ultraviolet spectrophotometer at 280 and 294·4 mμ. and, to allow correction for irrelevant absorption, at 340 and 370 mμ.

Copper sulphate specific gravity method. The $CuSO_4$ solutions were prepared from a stock solution of $D_{25}^{25} = 1{\cdot}1000$ (King, 1951), the technique described by Phillips *et al.* (1943) being otherwise strictly adhered to.

RESULTS

Tyrosine content of bovine serum proteins. From triplicate determinations on sera from twelve cattle, 1 mg. tyrosine is equivalent to $19{\cdot}78 \pm 1{\cdot}442$ mg. of total protein using the Greenberg method, and $19{\cdot}68 \pm 0{\cdot}663$ mg. of total protein using the technique of Goodwin & Morton. According to Snell & Snell (1937), 1 mg. tyrosine is contained in 19·2 mg. of bovine serum albumin or 20·0 mg. of serum globulin; assuming an albumin : globulin ratio of 2:3 for healthy cattle over 5 years old (Garner, 1950), 1 mg. tyrosine would be equivalent to 19·68 mg. of mixed protein. Greenberg's method (using the equivalent of 0·02 ml. of serum and 0·4 ml. of the standard tyrosine solution) may therefore be adapted to cattle serum by using the expression:

$$\text{Total protein (g./100 ml.)} = \frac{\text{Reading of test } (T)}{\text{Reading of standard } (S)} \times \frac{200 \times 0{\cdot}4}{1000} \times \frac{100}{0{\cdot}02} \times \frac{1}{1000} \times 19{\cdot}73 = \frac{T}{S} \times 7{\cdot}89.$$

The method of Goodwin & Morton would appear to be adaptable to the determination of serum proteins. The amount of irrelevant absorption in a 1 in 100 dilution of serum is negligible in the majority of cases: thus by measuring the extinction

at 280 and 294·4 mμ., the total protein content can be calculated from the expression

Total protein (g./100 ml.) = 9·76 ($2{\cdot}2E_{294.4} - E_{280}$).

Estimation of serum protein concentration from serum specific gravity. The relationship between the serum protein concentration (P, in g./100 ml.) and the serum specific gravity (G) is usually expressed in the form

$$P = a(G - b)$$

where a and b are constants.

Sera from 320 cattle have been examined, the serum protein values varying from 4·5 to 11·0 g./100 ml. and the specific gravities from 1·0200 to 1·0405. For the specific gravity range 1·0220 to 1·0365 (corresponding to a serum protein range of from 5·5 to 9·6 g.) the equation relating these quantities, obtained using values for 312 sera, was

$$P = 362{\cdot}0\ (G - 1{\cdot}0020)$$

($a = 362{\cdot}02 \pm 7{\cdot}668$, $b = 1{\cdot}00197 \pm 0{\cdot}000202$). Beyond these limits the relationship was no longer linear.

DISCUSSION

The value for the constant (1·0020) obtained for cattle in the equation relating serum protein concentrations and serum specific gravity is low compared with that found for the plasma of other species (Van Slyke, Hiller, Phillips, Hamilton, Dole, Archibald & Eder, 1950). The value for this constant should agree approximately with the specific gravity of a solution of the serum crystalloids. The sera were obtained from adult zebu cattle which were undoubtedly existing on a low plane of mineral nutrition, and it is possible that the low value of b reflects a lowered serum content of mineral salts.

SUMMARY

1. The tyrosine content of mixed bovine serum proteins has been determined. 1 mg. of tyrosine is contained in 19·78 mg. of total protein (tyrosine determined using the Folin & Ciocalteu reagent) or 19·68 mg. of total protein (tyrosine determined spectrophotometrically).

2. A linear relationship exists between the serum protein concentration and the serum specific gravity, provided that the latter falls within the range 1·0220 to 1·0365. Within these limits

$$P = 362{\cdot}0\ (G - 1{\cdot}0020).$$

Thanks are due to Mr J. D. Brewer for technical assistance.

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Reaction Between Hydrocyanic Acid, Cyanide Ion and Ferricytochrome *c*

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Potter & Lockhart (1939) reported that the enzymic reduction of cytochrome *c* by either the dihydrocozymase-diaphorase system or the succinic dehydrogenase system was blocked by 0·01 M-cyanide. In later communications (Lockhart & Potter, 1941; Potter, 1941), it was concluded that cytochrome *c* was involved in a reaction with cyanide. The evidence presented to support this conclusion can be summarized as follows. (1) By incubation of cytochrome *c* with cyanide, the enzymic reduction of the former was partly or completely prevented, depending on the cyanide concentration, and on the time and pH of incubation. The enzyme systems

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which are responsible for the reduction of cytochrome *c* were not affected. (2) Incubation with cyanide also shifted the absorption maximum of ferricytochrome *c* at 530 mμ. about 5 mμ. toward the red.

This conclusion was later confirmed by Horecker & Kornberg (1946), who studied this reaction in considerable detail and suggested that complex formation involved only the free cyanide ion. These workers demonstrated the reversibility of the reaction and determined the formation velocity constant and equilibrium constant from which they calculated the dissociation velocity constant. They also obtained values for the heat of dissociation and the energy of activation for the formation of the complex. The absorption spectrum of the ferricytochrome *c*-cyanide complex, obtained by Potter in the green region, was extended by these workers to the red. They found that the feeble bands of ferricytochrome *c* at 655 and 695 mμ. disappeared when it was converted to the complex.

In spite of the conclusive evidence put forward by these workers, the fact that cytochrome *c* forms a reversible complex with cyanide does not seem to have been generally accepted (see, for instance, Theorell, 1947; Wyman, 1948). In the present investigation, some of the results of Potter and of Horecker & Kornberg have been confirmed.

The formation velocity constant and the equilibrium constant of the reaction have been determined at different temperatures and over a wider pH range than hitherto investigated. Evidence is presented to show that ferricytochrome *c* is capable of reacting with both hydrocyanic acid and free cyanide ion; the former reaction predominates in acid solutions and the latter in neutral and slightly alkaline solutions. From the kinetic and equilibrium data forward and back velocity constants and the two equilibrium constants are calculated, together with heats and entropies of activation and the overall change in heat content and entropy associated with the formation of the complex. Where comparison is possible, the numerical values of those quantities differ from those reported by Horecker & Kornberg as would be expected because at the pH of their experiments, i.e. 7·4, the reactions with both hydrocyanic acid and cyanide ion are important, whereas they analysed their data in terms of a reaction with cyanide ion alone.

Experiments are also presented showing that the velocity constant for the formation of the complex decreases above pH 9·2, which may be attributed to an ionization in the ferricytochrome *c* molecule such that the formation of the complex occurs predominantly by reaction of the acid form.

It has been shown previously, however, that endogenous cytochrome *c*, i.e. cytochrome *c* as present in living cells and in certain colloidal enzyme preparations, does not react with cyanide (Tsou, 1951*b*). The present paper deals exclusively with exogenous cytochrome *c*.

EXPERIMENTAL

Cytochrome c of iron content 0·34% was prepared by the method of Keilin & Hartree (1945).

Heart-muscle preparation containing the enzymes responsible for the reduction of cytochrome *c* by succinate was prepared according to the Keilin & Hartree method as previously described (Tsou, 1951).

Cyanide. Neutral solutions were prepared each week and the cyanide concentration was determined by titration against standard silver nitrate solution immediately before use.

Following Latimer (1938), we have taken the results of Harman & Worley (1924) on the hydrolysis of KCN as the most reliable data for the ionization constant of HCN, K_a. From their values of the hydrolysis constant at various temperatures and the values for K_w, the ionic product of water, at temperatures 15–40° given by Harned & Owen (1943), we have calculated the values of K_a appropriate to the temperatures of our experiments. These are given later in Table 3. We have made no correction for ionic strength, partly because Harman & Worley's experiments were carried out at an ionic strength comparable to that employed in the present investigation. However, in calculating K_a from the hydrolysis constant a value of K_w corrected for ionic strength should strictly be used. But in view of the uncertainty in the hydrolysis data shown by the different results obtained by previous workers, e.g. Madsen (1901) gives a value for K_a of $4{\cdot}7 \times 10^{-10}$ at 18°, whereas the value calculated from Harman & Worley's data is about $2{\cdot}5 \times 10^{-10}$, and considering that the correction to K_w would only be of the order of 15%, we have chosen to neglect it. It must be borne in mind that values of velocity constants and equilibrium constants obtained later are subject to correction if a better value of K_a becomes available.

Buffers. Below pH 8·0, 0·075 M-phosphate buffers were used; and in the pH range 8·0–10·0, 0·075 M-borate buffers. pH measurements were made with a glass electrode and a Cambridge pH meter.

Light absorption. This was measured with a Beckman photoelectric spectrophotometer.

Estimation of reaction rate. The cytochrome *c* solution used was first acidified to about pH 3–4 and aerated to ensure complete oxidation. It was then titrated back to neutrality. This solution (1 ml.) was mixed with 5 ml. of 0·15 M-buffer of the required pH, the correct amount of neutralized cyanide, and water to make a total volume of 10 ml. (solution *A*). The pH of the solution was measured immediately at the end of the experiment at each temperature with a glass electrode assembly. Solution *B* was made up from 1 ml. of heart-muscle preparation, 5 ml. of 0·4 M-succinate and 0·25 M-phosphate buffer, pH 7·4, to a total volume of 50 ml. Periodically, 1 ml. portions of solution *A* were pipetted into 2 ml. portions of solution *B*. The optical density at 550 mμ. of such a mixture was read against a blank in a Beckman spectrophotometer until a maximum value was reached (usually after 10–30 sec.). The blank contained the same concentrations of all the reagents except cytochrome *c*. At this stage, the uncombined cytochrome *c* was fully reduced.

The molar concentration of free cytochrome *c*, [*c*], at any time *t* can be calculated from the following equation

$$[c] = \frac{D_t - D_\infty}{1{\cdot}88 \times 10^4} \times 3, \qquad \text{(i)}$$

where D_t is the steady density of the mixture prepared at time *t*; D_∞ is the density when all the cytochrome is converted to the cyanide complex prior to mixing with the heart-muscle preparation. This figure can also be obtained by

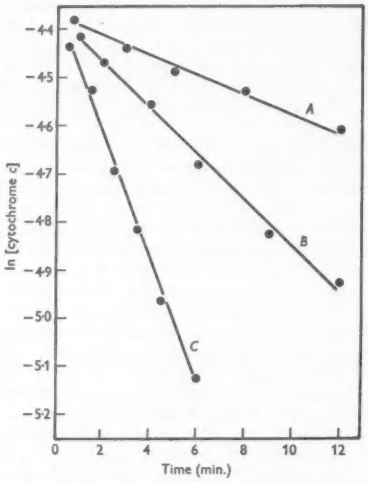

Fig. 1. Reaction of ferricytochrome *c* with cyanide. Plot of logarithm of (free cytochrome *c* concentration) against time, showing that the reaction follows the first-order equation

$$-\frac{2{\cdot}3\ \mathrm{d} \ln [c]}{\mathrm{d}t} = k_{\text{obs.}}[\mathrm{KCN}].$$

Temp. = 24·6°, pH = 7·4, and total cyanide concentrations were 4·2 (*A*), 10·3 (*B*), and 25·8 (*C*) mM respectively.

multiplying the total concentration of cytochrome *c* by $0{\cdot}92 \times 10^4$ which is the molecular extinction coefficient of the complex at 450 mμ. (Tsou, 1952); and $1{\cdot}88 \times 10^4$ is the difference between the molecular extinction coefficient of ferrocytochrome *c* (Theorell & Åkeson, 1941*a*) and that of the complex at 550 mμ. When KCN is present in excess, the reaction is first order and the velocity constant is calculated from

$$k_{\text{obs.}} = -\frac{2{\cdot}3}{[\mathrm{KCN}]} \times \frac{\mathrm{d} \ln [c]}{\mathrm{d}t}, \qquad \text{(ii)}$$

where [KCN] is the concentration of total cyanide and d log [*c*]/d*t* was obtained graphically by plotting ln [*c*] against time (Fig. 1).

Estimation of equilibrium constant. The same method is also available for estimation of the equilibrium constant of the reaction between cytochrome *c* and cyanide, except that lower cyanide concentrations and longer incubation times have to be used. Precautions to prevent loss of HCN vapour were necessary (Horecker & Kornberg, 1946).

RESULTS

Order of reaction. In presence of excess cyanide, the reaction followed the first-order Eqn. ii, as is shown in Fig. 1. These first-order constants, $k_{\text{obs.}}$, were found to depend on the hydrogen-ion concentration, as shown in Fig. 2, where the values obtained at 11·7 and 24·6° are plotted as a function of pH over the range 6·0–10·0. Similar results were obtained at 18·2 and 30·4° for the pH range 6·0–8·0.

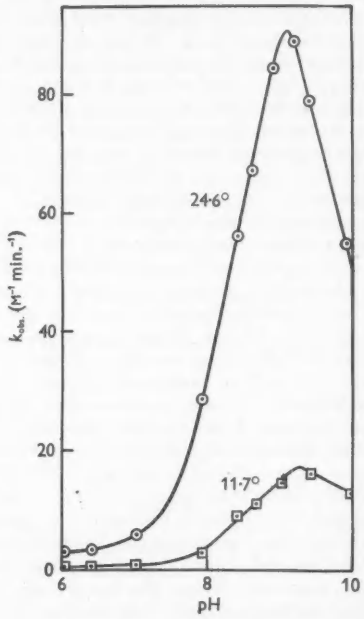

Fig. 2. Variation of the observed velocity constant for the formation of the ferricytochrome *c*-cyanide complex with the hydrogen-ion concentration, at 11·7 and 24·6°, in 0·075 M-phosphate and borate buffers.

Equilibrium measurements. Using the method described above, the equilibrium concentration of the ferricytochrome *c*-cyanide complex in solutions containing ferricytochrome *c* and potassium cyanide was measured at several potassium cyanide concentrations over the pH range 5·8–7·5. From the results an equilibrium constant K_{KCN} was calculated for the overall reaction from the equation

$$K_{\text{KCN}} = \frac{[\text{cyt. } c\text{-CN}]}{[\text{cyt. } c] \times [\mathrm{KCN}]}. \qquad \text{(iii)}$$

It will be shown below that this overall equilibrium constant is compounded of two equilibrium constants appropriate to the two paths the reaction can take, one involving the un-ionized HCN molecule

and the other involving the free cyanide ion. By expressing the experimental results in this form, i.e. Eqn. iii, they are more amenable to analysis. Values of this equilibrium constant over the pH range 5·8–7·5, at 13·8 and 24·6°, are recorded in Tables 1 and 2.

Table 1. *Equilibrium constant data at 13·8° for the formation of the ferricytochrome c-cyanide complex calculated from Eqn. iii at different hydrogen-ion concentrations*

pH	KCN (mM)	K_{KCN} ($\times 10^{-3}$ M)	Mean K_{KCN} ($\times 10^{-3}$ M)
5·86	2·9	0·194	0·200
	0·97	0·206	
6·30	1·95	0·526	0·513
	0·65	0·500	
6·74	0·97	1·61	1·48
	0·32	1·35	
6·98	0·65	2·47	2·38
	0·22	2·29	
7·41	0·44	5·55	5·30
	0·22	5·05	

Table 2. *Equilibrium constant data at 24·6°*

(K_{KCN} values are calculated from Eqn. iii.)

pH	KCN (mM)	K_{KCN} ($\times 10^{-3}$ M)	Mean K_{KCN} ($\times 10^{-3}$ M)
5·83	2·9	0·319	0·323
	0·97	0·327	
6·25	1·95	0·952	0·889
	0·65	0·826	
6·72	0·65	2·14	2·15
	0·22	2·16	
6·98	0·44	4·42	4·36
	0·22	4·30	
7·46	0·33	14·3	13·0
	0·16	11·6	

ANALYSIS OF THE RESULTS

An examination of the hydrogen-ion dependence of the bimolecular velocity constant for the formation of the complex given in Fig. 2 shows that at high hydrogen-ion concentrations, up to about pH 6·0, the velocity constant is independent of pH and has a low value, but that as the solution is made more alkaline, pH 7·0–9·0, the velocity constant increases rapidly. This suggests that in the pH range 6·0–9·0 we are dealing with two reactions giving the complex, the first with HCN and the second with CN^-:

$$\text{cyt. } c + HCN \overset{k_{HCN}}{\rightleftharpoons} \text{cyt. } c.CN + H^+, \qquad (1)$$

$$\text{cyt. } c + CN^- \overset{k_{CN^-}}{\rightleftharpoons} \text{cyt. } c.CN. \qquad (2)$$

Representing the individual velocity constants for these two reactions by k_{HCN} and k_{CN^-}, it can be shown that the observed bimolecular velocity constant, $k_{obs.}$, should be given by

$$k_{obs.} = \frac{k_{HCN}.[H^+]}{[H^+]+K_a} + \frac{k_{CN^-}.K_a}{[H^+]+K_a}, \qquad (iv)$$

since the fraction of un-ionized HCN and the cyanide ion at any hydrogen-ion concentration are given by $\frac{[H^+]}{[H^+]+K_a}$ and $\frac{K_a}{[H^+]+K_a}$, respectively, where K_a is the ionization constant of HCN. Thus, to test the mechanism given by reactions 1 and 2 above, $k_{obs.}([H^+]+K_a)$ can be plotted against $[H^+]$, for, if

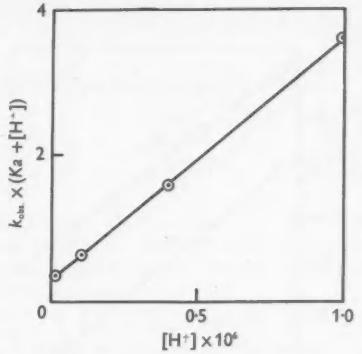

Fig. 3. Plot of $k_{obs.}([H^+]+K_a)$ against hydrogen-ion concentration for the data at 24·6° in Fig. 2.

Eqn. iv holds, the graph should be linear. Fig. 3, which is the plot for the data at 24·6°, shows that this is so. Similar linear plots were obtained for the data at 11·7, 18·2 and 30·4°. This linearity supports the mechanism given by reactions 1 and 2 and the further analysis rests on this basis.

The slope of the line in Fig. 3 gives the value of $k_{HCN} = 3{\cdot}26 \pm 0{\cdot}02\ \text{M}^{-1}\ \text{min.}^{-1}$ and the intercept could be used to obtain a value of the product $k_{CN^-}.K_a$. However, a better value for this quantity with more accurately determined limits can be got by an alternative plot of the data. The function $k_{obs.}([H^+]+K_a)\ [H^+]$ plotted against the reciprocal of $[H^+]$ is also linear and the slope in this case is given by $k_{CN^-}.K_a$. Using a value of K_a calculated as described above, k_{CN^-} is found to be $914 \pm 24\ \text{M}^{-1}$ sec.$^{-1}$ at 24·6°. The formation of the complex from the free cyanide ion thus proceeds about 280 times faster than the formation from the undissociated HCN molecule at this temperature. The calculated values of K_a and the values for k_{HCN} and k_{CN^-} at the four temperatures used are listed in Table 3.

Fig. 4 shows the appropriate plot to determine the activation energy E for the two reactions. The vertical lines within the circles in this diagram represent the spread of the experimental values.

Table 4 records values of these activation energies together with A, the temperature-independent factor, and ΔH^* and ΔS^*, the heat and entropy of activation for the two reactions. The value of A is obtained from the equation $k = Ae^{-E/RT}$; the heat of activation is given by $\Delta H^* = E - RT$ and the entropy of activation is calculated from the data at 24·6° using the equation

$$k = 1{\cdot}43 \times 10^{13} \exp\left(\frac{\Delta S^*}{R}\right) \exp\left(\frac{\Delta H^*}{RT}\right). \quad \text{(v)}$$

Examination of Fig. 2 now shows that the above mechanism for the formation of the complex does not hold over the entire pH range examined. If it did so, in alkaline solution when all the cyanide is present as CN^-, $k_{obs.}$ should tend asymptotically to the value of k_{CN^-}. It is clear that another ionization is operative, responsible for a diminution in the velocity of function of the complex. The ionization of HCN has already been taken into account, so this additional ionization must be attributed to the ferricytochrome c. The position of the maximum in Fig. 2 indicates roughly that the pK for this ionization lies between 8·5 and 9·2, having a bigger value the lower the temperature.

The simplest mechanism based on an ionization of this kind is to assume that the acid form of ferricytochrome c reacts with HCN and CN^-, giving the complex, and that the alkaline form does not react at all. If K_F is the ionization constant of ferricytochrome c, then the fraction of the acid form at any hydrogen-ion concentration will be given by $\frac{[H^+]}{K_F + [H^+]}$, and it follows that the relation between $k_{obs.}$, the observed velocity constant for the formation of the complex, and $k_{calc.}$, the value this velocity constant would have if the ionization did not occur, will be given by

$$k_{obs.} = k_{calc.} \frac{[H^+]}{K_F + [H^+]}. \quad \text{(vi)}$$

According to this equation, $k_{calc.}/k_{obs.}$ plotted against the reciprocal of $[H^+]$ should be linear. Fig. 5 shows the data for 24·6° and 11·7° plotted in this way. Quite good straight lines are obtained which thus support this simple ionization mechanism and it may be concluded that the assumption that

Table 3. *Velocity constants for ferricytochrome* c-*cyanide complex formation and ionization constants for* HCN

Temp. (°)	$K_a \times 10^{10}$	k_{HCN} (M^{-1} min.$^{-1}$)	k_{CN^-} (M^{-1} min.$^{-1}$)
11·7	1·64	0·825±0·015	253±12
18·2	2·51	1·72 ±0·03	465±15
24·6	3·57	3·26 ±0·02	914±24
30·4	5·21	6·28 ±0·04	1560±30

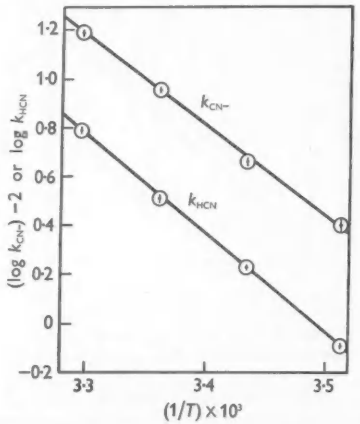

Fig. 4. Plots to determine the activation energies for the formation of the ferricytochrome c-cyanide complex from the free cyanide ion (upper line) and the undissociated hydrocyanic acid molecule (lower line).

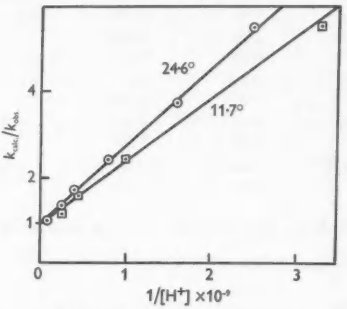

Fig. 5. Plot of the calculated velocity constant for the formation of the complex divided by the observed value as a function of $1/[H^+]$ at 11·7 and 24·6° based on the data in Fig. 2.

Table 4. *Kinetic data for ferricytochrome* c-*cyanide complex formation*

(For definition of symbols, see Eqn. v.)

Reacting species	k at 24·6° (M^{-1} sec.$^{-1}$)	E (kg.cal.)	A (M^{-1} sec.$^{-1}$)	ΔH^* (kg.cal.)	ΔS^* (e.u.)
HCN	0·0543	18·4±0·4	$2{\cdot}2 \times 10^{12}$	17·8±0·4	−5·9±1·4
CN^-	15·2	17·0±0·5	$5{\cdot}4 \times 10^{13}$	16·4±0·5	0·5±1·7

Table 5. *Acidity constants, heats and entropies of ionization for certain groups in proteins as compared with those of an ionizing group present in ferricytochrome c*

Group	Reference	pK	Temp. (°)	ΔH (kg.cal.)	ΔS (e.u.)
Phenolic OH (tyrosine)	1	9·8–10·4	25	6·0	−24·4 to −27·0
Glyoxaline (histidine)	1	5·6–7·4 (8·5)	25	6·9 to 7·5	−0·3 to −10·3 (−15·3)
Glyoxaline (haemin-linked)	2	9·5	25	6·2	−22·7
Haemin-water (in metmyoglobin)	3	8·85	25	3·85±0·4	−27·0±2
Group in ferricytochrome *c*	4	8·75	24·6	2·9±0·7	−30±3

(1) Cohn & Edsall (1943). The limit 8·5 in the pK for histidine-glyoxaline is taken from Theorell & Åkeson (1941*b*). The ΔH and ΔS values are for both amino-acids and peptides and in general are experimental values at various ionic strengths.
(2) Russell & Pauling (1939). The ΔH value is that used by Wyman (1948).
(3) George & Hanania (unpublished results). The figures refer to an ionic strength similar to that used in the present investigation. The true thermodynamic values at 25° are nearer pK = 8·99, $\Delta H = 5·9 \pm 0·7$ kg.cal. and $\Delta S = -21 \pm 2$ e.u.
(4) Present study.

Table 6. *Derived kinetic data for the dissociation of the ferricytochrome c-cyanide complex*

Dissociation into free cyt. +	k, at 24·6° (sec.$^{-1}$)	E (kg.cal.)	A	ΔH^* (kg.cal.)	ΔS^* (e.u.)
HCN	$1·13 \times 10^2$ M^{-1}	9·2±1·0	$7·1 \times 10^8$	8·6±1·0	−21·6±3·0
CN^-	$1·25 \times 10^{-5}$	15·9±1·0	$6·25 \times 10^6$	15·3±1·0	−30·8±3·0

the reaction of the alkaline form of ferricytochrome *c* giving the complex can be neglected was justified.

From Eqn. vi it can be seen that the slopes of these two lines give the values of K_F at the two temperatures. The values are $1·43 \pm 0·07 \times 10^{-9}$ and $1·78 \pm 0·02 \times 10^{-9}$ at 11·7 and 24·6°, respectively, corresponding to pK values of $8·84 \pm 0·03$ and $8·75 \pm 0·01$. Table 5 gives the heat and entropy of ionization calculated from these data, together with comparable data for the ionization of groups known to occur in proteins, and for the water molecule bound to the haemin group in metmyoglobin (see Eqn. 3).

$$Fe^+(H_2O) \rightleftharpoons FeOH + H^+. \qquad (3)$$

Using the values of k_{HCN} and k_{CN^-} obtained above, the equilibrium data recorded in Tables 1 and 2 can be analysed in the following way. The overall equilibrium constant K_{KCN} will be given by the equation

$$K_{KCN} = \frac{\dfrac{k_{HCN} \cdot [H^+]}{K_a + [H^+]} + \dfrac{k_{CN^-} \cdot K_a}{K_a + [H^+]}}{k_{diss.}}, \qquad (vii)$$

where $k_{diss.}$ is the velocity constant for the dissociation of the complex. The three constants in the numerator are known, and its numerical value has been calculated for each pH value in Tables 1 and 2. This divided by the respective value of K_{KCN} gives the corresponding value of $k_{diss.}$. In Fig. 6, $k_{diss.}$ obtained in this way is plotted against $[H^+]$. The straight lines obtained show that $k_{diss.}$ is compounded of a hydrogen ion-dependent dissociation and a normal dissociation. Inspection of reactions (1) and (2) shows that this was to be expected, for the corresponding back reactions would have these characteristics. If k'_{HCN} and k'_{CN^-} represent the velocity constants for these back reactions, k'_{HCN} being the velocity constant at unit hydrogen-ion concentration, then

$$k_{diss.} = k'_{CN^-} + k'_{HCN}[H^+]. \qquad (viii)$$

The intercepts and slopes in Fig. 6 can thus be identified with k'_{CN^-} and k'_{HCN}. These constants have the values $2·7 \times 10^{-4}$ min.$^{-1}$ and $7·5 \times 10^{-4}$ min.$^{-1}$ at

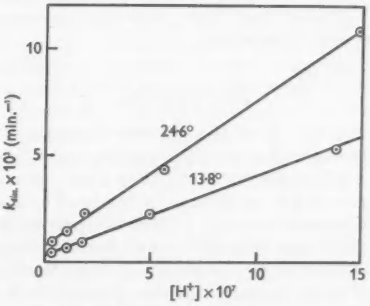

Fig. 6. Plot of the calculated dissociation velocity constant as a function of $[H^+]$ at 13·8 and 24·6° based on kinetic data, including that in Fig. 2, and the equilibrium data recorded in Tables 1 and 2.

13·8 and 24·6°, and $3·78 \times 10^3$ M^{-1} min.$^{-1}$ and $6·8 \times 10^3$ M^{-1} min.$^{-1}$ at the same two temperatures, respectively. Table 6 lists the energies of activation, temperature-independent factors, heats and entropies of activation, calculated from these values in the same way as that used for the formation velocity constant data in Table 4. The individual equilibrium constants for reactions (1) and (2)

can now be calculated, for $K_1 = k_{HCN}/k'_{HCN}$ and $K_2 = k_{CN^-}/k'_{CN^-}$. In addition the change in heat content, ΔH, and the entropy change associated with the two reactions, may be obtained from the difference between the heats and entropies of activation of the forward and back reactions respectively. These data are listed in Table 7.

Table 7. *Derived equilibrium constants and overall heat and entropy changes for the formation of the ferricytochrome* c*-cyanide complex at* 24·6°

Reacting species	Equilibrium constants	ΔH (kg.cal.)	ΔS (e.u.)
HCN	$4{\cdot}8 \times 10^{-4}$	$9{\cdot}2 \pm 1{\cdot}4$	$+15{\cdot}7 \pm 4{\cdot}4$
CN^-	$1{\cdot}22 \times 10^6$ M^{-1}	$1{\cdot}1 \pm 1{\cdot}5$	$+31{\cdot}3 \pm 4{\cdot}7$

The complicated nature of the overall reaction revealed by this analysis shows that it would not have been possible to obtain reliable quantitative data from measurements of the equilibrium constant alone over a range of pH. Combining Eqn. vii with Eqn. viii the full expression for the overall equilibrium constant is

$$K_{KCN} = \frac{\dfrac{k_{HCN} \cdot [H^+]}{K_a + [H^+]} + \dfrac{k_{CN^-} \cdot K_a}{K_a + [H^+]}}{k'_{HCN} \cdot [H^+] + k'_{CN^-}}.$$

In acid solution this expression reduces to k_{HCN}/k'_{HCN} and so a value of K_1 could be obtained, but in solutions sufficiently alkaline to assume that K_1 is negligible, the ionization of the ferricytochrome *c* itself intervenes. At intermediate pH values a knowledge of individual velocity constants is essential for the analysis.

DISCUSSION

Horecker & Kornberg (1946) determined the velocity constants and the equilibrium constants over a pH range of 7·4–8. They found that these constants, when calculated on the basis of cyanide ion, remained constant within this pH range and they concluded, therefore, that ferricytochrome *c* reacted exclusively with cyanide ion. Results obtained in the present study have shown that ferricytochrome *c* reacts with both hydrocyanic acid and cyanide ion, the velocity constant for the latter reaction being about 280 times as large as that for the former reaction. The reaction with hydrocyanic acid is significant only at those pH values where cyanide is present predominantly as undissociated hydrocyanic acid.

Horecker & Kornberg's values for the velocity constant, the equilibrium constant and the activation energy for the cyanide ion reaction do not agree with ours. In part this is due to not taking into account the reaction with undissociated hydrocyanic acid and in part to using a different value for the ionization constant of hydrocyanic acid. They do not state explicitly what value they have used, but in the caption to one of their figures they gave the free cyanide ion concentration at pH 7·4 in $4{\cdot}5 \times 10^{-3}$ M-potassium cyanide as $7{\cdot}9 \times 10^{-5}$ M-cyanide ion at 24°. This corresponds to a value for the ionization constant of hydrocyanic acid of $7{\cdot}0 \times 10^{-10}$ M in accord with the data of Madsen (1901). Using this and their value of 550 M^{-1} min.$^{-1}$ for k_{CN^-}, it can be shown that their experimental velocity constant would be about 9·5 M^{-1} min.$^{-1}$, whereas our data lead to a value of 11·4 M^{-1} min.$^{-1}$. There is thus far less numerical discrepancy between the experimental data than would appear from the determined constants, e.g. $k_{CN^-} = 914$ and 550 M^{-1} min.$^{-1}$ in our analysis and theirs, respectively. Their value for the activation energy, however, is widely different from ours, i.e. 26·1 kg.cal. as compared with our value of 17 kg.cal. We do not understand why this is so, except that it would arise if the fraction of free cyanide ion in potassium cyanide solution had been taken as independent of temperature. The difference between the activation energies corresponds closely to the heat of ionization of hydrocyanic acid of 9·8 kg.cal. (Madsen, 1901) or 10·8 kg.cal. (Harman & Worley, 1924) required on this supposition. The discrepancy between the values for the equilibrium constant, K_2, can also be accounted for in a similar way.

There is a very simple relationship which should hold between the two equilibrium constants for the formation of a complex through an ion and the corresponding undissociated acid. In the case of ferricytochrome *c* the cyanide complex is formed by reactions (1) and (2) (see p. 443). From the expressions for the two equilibrium constants, K_1 and K_2, it follows that $K_1/K_2 = K_a$, where K_a is the ionization constant for hydrocyanic acid. In Table 8 the

Table 8. *Relation between the equilibrium constants of the cyanide and hydrocyanic acid reactions with ferricytochrome* c *and the ionization constant of hydrocyanic acid*

Temp. (°)	13·8	24·6
Equilibrium constant of the HCN reaction, $K_1 \times 10^4$	2·72	4·8
Equilibrium constant of the CN^- reaction, $K_2 \times 10^{-6}$	1·12	1·22
$K_1/K_2 \times 10^{10}$	2·43	3·93
Ionization constant of HCN $\times 10^{10}$	1·90	3·57

values of K_1/K_2 from the data obtained at 13·8 and 24·6° are compared with K_a. The agreement is very satisfactory in view of the lengthy derivation of the two equilibrium constants. The differences between the overall heats and entropies of the formation of the complex which are listed in Table 7 are also a consequence of this relationship between the two equilibrium constants. The differences should

correspond to the heat and entropy of ionization of hydrocyanic acid, i.e. $\Delta H = 10{\cdot}8$ kg.cal. and $\Delta S = +6{\cdot}4$ e.u. (entropy units), which can be seen to be the case within the rather wide limits of the experimental uncertainty associated with these derived quantities.

The peculiar features of the formation of this cyanide complex can best be shown by the data for its formation from the cyanide ion by reaction (2). It is formed in a slightly endothermic reaction, $\Delta H = 1{\cdot}1 \pm 1{\cdot}5$ kg.cal. (Table 7), yet the activation energies for the forward and back reactions are quite large, $17{\cdot}0 \pm 0{\cdot}5$ and $15{\cdot}9 \pm 1{\cdot}0$ kg.cal. respectively (Tables 4 and 6). An activation energy of this magnitude is not unreasonable for the back reaction which involves the breaking of a Fe-CN bond, but the high activation energy for the forward reaction suggests that a strong bond has to be broken to allow the cyanide complex to form.

The overall entropy of formation of the complex in reaction (2) has a very large positive value, $\Delta S = 31{\cdot}3 \pm 4{\cdot}7$ e.u. (Table 7). Now in this reaction the cyanide ion is bound, and some part of its total entropy of $+25$ e.u. will be lost (Latimer, 1938). This might easily be about 10 e.u. and so, when the cyanide complex forms, an entropy change of about $+40$ e.u. must arise from changes in structure in the ferricytochrome *c* itself. This is very suggestive that the haematin is bound on both sides of the iron atom, for the high activation energy referred to above would be required to free one of the iron valencies, and the large positive entropy change would result from the more random arrangement of that part of the protein molecule previously bound to the iron atom. Such a model for cytochrome *c* has been discussed by Theorell (1941).

The dissociation of the complex is necessarily attended by a large negative entropy of activation, $-30{\cdot}8 \pm 3{\cdot}0$ e.u. (Table 6). This contributes to a great extent to the low value for the dissociation velocity constant, which has a low temperature-independent factor of $6{\cdot}25 \times 10^6$, compared with those for normal reactions of about 10^{13}. In fact the existence of the complex can be regarded as a consequence of this low dissociation velocity constant. The formation velocity constant, apart from the high activation energy, is normal in that the temperature-independent factor is about 10^{13} (Table 4). This reaction may be contrasted with other haemoprotein reactions such as that between haemoglobin and oxygen, when the existence of oxyhaemoglobin is not a consequence of a slow dissociation velocity constant but of an extremely large formation velocity constant. It is an attractive hypothesis to suppose that the orientation and binding of the haem or haematin groups is responsible for these differences. Hanania, George & Irvine (unpublished results) have recently observed that the formation of the metmyoglobin-cyanide complex is comparatively slow. The observed bimolecular constant at pH 7·0 and 18° is about $80\,\text{M}^{-1}\,\text{sec.}^{-1}$ compared with the value $0{\cdot}037\,\text{M}^{-1}\,\text{sec.}^{-1}$ for ferricytochrome *c* under the same conditions. This suggests that simple structural considerations alone cannot account for differences in reactivity.

Kinetic studies in alkaline solutions indicate that the reaction between ferricytochrome *c* and cyanide is affected by an ionizing group in the vicinity of the prosthetic group of the former. The dissociated form of ferricytochrome *c* reacts with cyanide, if at all, much more slowly than does undissociated ferricytochrome *c* with cyanide ion.

This ionizing group in ferricytochrome *c* which affects the formation velocity of the cyanide complex is of particular interest in connexion with the binding of the haemin-iron atom to the protein. No ionizing group with precisely the same pK value has been revealed in other investigations. By oxidation-reduction potential studies, Rodkey & Ball (1950) discovered an ionizing group with a pK value of 7·8 and by spectrophotometric titration, Theorell & Åkeson (1941*b*) showed that an ionizing group, with a pK of 9·35, is responsible for the change in absorption spectrum of ferricytochrome *c* from types III to IV. The pK value obtained in the present study is sufficiently different from that of Rodkey & Ball to exclude identification of the two groups concerned. On the other hand, it seems to be possible that the ionizing group responsible for the change in absorption spectrum obtained by Theorell & Åkeson might be the same group described in the present work. The discrepancy in pK values could be attributed to the differences in ionic strength of the ferricytochrome *c* solutions used, and it is not inconceivable that cytochrome *c* preparations with an iron content of 0·34 % used in the present work might behave slightly differently from the preparation of iron content 0·43 % employed by Theorell & Åkeson.

By titration studies, Theorell & Åkeson (1941*c*) reached the conclusion that only one of the three histidine molecules present in cytochrome *c* was titrated within its normal pH range and two haemin-linked acid groups were titrated between pH 9 and 10. It was argued therefore that these two haemin-linked groups were both histidine glyoxaline groups. This theory has also received the support of Paul, who succeeded in splitting off the cytochrome *c* prosthetic group from the protein (Paul, 1950), and in a brief communication (Paul, 1949) also reported that between pH 4 and 6 two more acid groups were titrated in the protein residue than in the intact cytochrome molecule. However, no details of the latter findings have yet been made available.

Unfortunately, the heat of ionization of Theorell & Åkeson's haemin-linked acid groups was not

reported. The heat and entropy of ionization of the group described in the present work seem to be rather different from what would be expected from a histidine glyoxaline residue, although it is not known whether haemin-linked glyoxaline groups might have slightly different values. The pK value of 9·5 reported by Russell & Pauling (1939) for glyoxaline in the methaemoglobin-glyoxaline complex is an indication that this may be so, but again, the values of ΔH and ΔS are not available, and so a full comparison is not yet possible. It is interesting to point out, however, that the heat of ionization for the group in cytochrome *c* is particularly low, i.e. $2{\cdot}9 \pm 0{\cdot}7$ kg.cal., whereas weak acids with a pK in this range usually have heats of the order of 10–12 kg.cal. (Cohn & Edsall, 1943).

SUMMARY

1. A kinetic investigation of the formation of the ferricytochrome *c*-cyanide complex over the pH range 6·0–9·0 suggests that both un-ionized hydrocyanic acid and the free cyanide ion react:

$$\text{Cyt. } c + \text{HCN} \rightleftharpoons \text{Cyt. } c\text{-CN} + \text{H}^+, \quad (1)$$

$$\text{Cyt. } c + \text{CN}^- \rightleftharpoons \text{Cyt. } c\text{-CN}. \quad (2)$$

Analysing the data according to this mechanism, the bimolecular constants k_{HCN} and k_{CN^-} were found to be $5{\cdot}43 \times 10^{-2}$ and $15{\cdot}2\,\text{M}^{-1}\,\text{sec.}^{-1}$ respectively, at 24·6°. From experimental data at various temperatures, the activation energies were determined as $18{\cdot}4 \pm 0{\cdot}4$ and $17{\cdot}0 \pm 0{\cdot}5$ kg.cal. respectively.

2. Using these velocity constants and equilibrium data obtained over the pH range 5·8–7·5 the dissociation velocity constants were calculated and found to be of the form

$$k_{\text{diss.}} = k'_{\text{CN}^-} + k'_{\text{HCN}}\,[\text{H}^+],$$

where k'_{CN^-} and k'_{HCN} can be identified as the reverse of reactions (2) and (1) respectively. At 24·6°, k'_{CN^-} and k'_{HCN} are $1{\cdot}25 \times 10^{-5}\,\text{sec.}^{-1}$ and $1{\cdot}13 \times 10^{3}\,\text{M}^{-1}\,\text{sec.}^{-1}$ respectively and the activation energies $15{\cdot}9 \pm 1{\cdot}0$ and $9{\cdot}2 \pm 1{\cdot}0$ kg.cal.

3. Combining these kinetic data gives the values for the equilibrium constants for reactions (1) and (2), $K_1 = 4{\cdot}8 \times 10^{-4}$ and $K_2 = 1{\cdot}22 \times 10^{6}\,\text{M}^{-1}$. The corresponding changes in heat content and entropy are $9{\cdot}2 \pm 1{\cdot}4$ kg.cal. and $+15{\cdot}7 \pm 4{\cdot}4$ e.u.; and $1{\cdot}1 \pm 1{\cdot}5$ kg.cal. and $+31{\cdot}3 \pm 4{\cdot}7$ e.u. respectively. For such reactions the relationship $K_1/K_2 = K_a$ should hold, where K_a is the dissociation constant of hydrocyanic acid: this was found to be so within the experimental error.

4. Kinetic experiments in alkaline solutions, pH 9–10, showed that the velocity constant for the formation of the complex decreases, which may be attributed to an ionization on the ferricytochrome *c* molecule such that the formation of the complex occurs predominantly by reaction of the acid form.

5. The pK for this ionization was calculated as 8·75 at 24·6° and the heat and entropy of ionization were found to be $2{\cdot}9 \pm 0{\cdot}7$ kg.cal. and -30 ± 3 e.u. respectively.

6. The quantitative data given above support a model for ferricytochrome *c* in which the haematin iron atom is firmly bound on both sides to the protein.

We wish to express our gratitude to Prof. D. Keilin, F.R.S., for his constant interest and advice in this work.

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Fractionation of the Nitrogenous Water-soluble Constituents of Liver

1. THE ISOLATION OF GLYCERYLPHOSPHORYLETHANOLAMINE AND OF TAURINE

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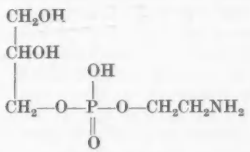
Any theory of protein synthesis *in vivo* has to take into account the apparent absence from the cell of the obvious intermediates in the synthesis of protein from amino-acids, namely peptides. Linderstrøm-Lang (1949) has pointed out that thermodynamic considerations would render unlikely the accumulation of appreciable quantities of peptides of low molecular weight; nevertheless, such compounds might be traced by the improved methods now available for the fractionation of amino-acids and peptides.

It was considered worth while to re-open this question by applying to the water-soluble constituents of liver the methods of fractionation used by us in the isolation of the toxic factor from agenized flour (Campbell, Work & Mellanby, 1951). Liver was chosen as an organ with a rapid rate of metabolism and a considerable capacity for protein synthesis. This paper presents some preliminary results on the fractionation of liver extracts during which we have isolated and characterized glycerylphosphorylethanolamine, (I), a substance not



(I)

α-Glycerylphosphorylethanolamine

previously known as a natural metabolite. We have also isolated taurine and have identified various amino-acids as normal constituents of liver cells. A preliminary account of this work has already been published (Campbell, Simmonds & Work, 1951).

EXPERIMENTAL

Chromatography. Paper-partition chromatography was carried out as described by Consden, Gordon & Martin (1944). The following solvents were used: phenol/NH_3 with KCN (Dent, 1948); 'collidine' (Dent, 1948); butanol/acetic acid/water in proportions given by Campbell, Work & Mellanby (1951); pyridine/amyl alcohol/water, 35:35:30% (v/v) as described by Edman (1945); and propanol/NH_3 (Hanes & Isherwood, 1949).

Liver extracts. Pig liver was placed in ice immediately after removal from the animal and was homogenized at 0° in a blender with the minimum quantity of water. The pH of the homogenate was adjusted to about 5 by addition of 3N-HCl and the whole homogenate (from 3012 g. liver) poured into 40 l. of boiling distilled water. The temperature was held at 100° for 30 min. and the coagulated protein then removed by filtration. The filter cake was resuspended in 10 l. of boiling water and again separated and the process repeated twice with 5 l. of water. The combined extracts were concentrated to 5600 ml. at 50–60° and filtered. The filtrate contained 11·35 g. N (Kjeldahl).

The concentrated extract was dialysed against 20 l. of distilled water for 24 hr. and then against further lots of about 7 l. The combined diffusates contained 6·45 g. N.

PRELIMINARY FRACTIONATION

Zeo-Karb. A column of Zeo-Karb 225 (The Permutit Co. Ltd., London, W. 4), diameter 2·25 in., was prepared with 200 g. (dry wt.) of size-graded resin (200 mesh/in. to 250 mesh/in. grid). The column was activated with 5N-HCl as described by Partridge & Westall (1949) for Zeo-Karb 215. The liver diffusate (3 g. N) was added to the column and the effluent (fraction *A*) collected (total N = 1090 mg.). The diffusate was washed into the column with distilled water and washing continued until a drop of the effluent failed to give a blue colour on paper sprayed with ninhydrin. The material still adsorbed on the column was displaced with an aqueous solution of NH_3 (0·2M) and the effluent collected in bulk (fraction *B*, total N = 1430 mg.). This fraction was then applied to filter paper and chromatographed in phenol/NH_3 and collidine. After development with ninhydrin the substances present were identified by comparison with Dent's map (Dent, 1948).

Mercury precipitation. Fraction *A* (3440 ml.) was found by paper chromatography to contain at least three substances giving coloured products with ninhydrin. These three substances were all precipitated as their mercury salts by the method of Neuberg & Kerb (1912). To 100 ml. of *A* was added, in 1 ml. portions alternately, a 25% (w/v) solution of mercuric acetate (25 ml.) and 10% (w/v) Na_2CO_3 (25 ml.). Absolute ethanol (250 ml.) was then added and the mixture left at 0° for 2 hr. The precipitate was collected in a centrifuge and washed twice with 66% ethanol (60 ml. total). The combined washings were added to the supernatant (fraction *C*, 14·5 mg. N). The precipitate was suspended in water (400 ml.) and decomposed with H_2S (fraction *D*, 11·2 mg. N in 150 ml.). Paper chromatography of fraction *C* showed that it was almost free of ninhydrin-positive material, whereas fraction *D* (50 μl.), run on paper

chromatograms and sprayed with ninhydrin, showed three well defined spots which we shall refer to as α, β and γ, in the order of increasing R_F values in amyl alcohol/pyridine.

Properties of three acidic compounds, α, β and γ. As the three compounds, in fraction *D* all passed through a Zeo-Karb 225 column they must be strong acids, but they also gave a blue colour with ninhydrin and presumably possessed primary NH_2 groups. A sample of *D* was hydrolysed with 6N-HCl for 16 hr. at 110° and amino N before and after hydrolysis measured by the Van Slyke HNO_2 method. There was no increase in amino N. Samples of *D* before and after hydrolysis were run on two-dimensional paper chromatograms using phenol/NH_3 and 'collidine' as solvents. Spots α and β disappeared on hydrolysis, spot γ was unaffected. In the hydrolysed material a new spot appeared in the position normally occupied by ethanolamine. As *D* contained phosphorus, a sample of ethanolamine phosphoric acid was synthesized by the method of Outhouse (1937) and compared with the three unknown substances. In no solvent could α be separated from authentic ethanolamine-*O*-phosphoric acid.

The position of γ on a two-dimensional chromatogram suggested that it might be taurine, and addition of an authentic sample of taurine to a chromatogram showed that it could not be separated from γ. This preliminary identification of γ as taurine was confirmed by isolation and analysis (see p. 452).

Substance β appeared (phenol/collidine) in the position reported by Dent (1948) as occupied by citrulline, but it was unaffected by pretreatment of the paper with basic copper carbonate (Crumpler & Dent, 1949) and disappeared on acid hydrolysis.

Isolation of acid-labile substance β from liver

Since the nature of β could not be deduced from its behaviour on chromatograms it became necessary to isolate a pure sample. Fraction *D* containing taurine, ethanolaminephosphoric acid and β was a suitable starting material for this project; ethanolaminephosphoric acid was a minor constituent as compared to the two other substances.

Precipitation with heavy metals. Attempts were made to separate substance β from fraction *D* as the Cu salt, but none of the methods tried was successful. The uranium acetate method of Colowick & Cori (1939) for the precipitation of phosphate esters also failed. Au and Pt salts were also tried without success. Separation by the Cd salt technique of Baer & Kates (1948*a*) also failed.

Fractionation on anion-exchange resin. A column was prepared with 0·5 g. of Deacidite F (The Permutit Co. Ltd.), a strongly basic anion-exchange resin, and a sample of fraction *D* added. The effluent from the column failed to give any reaction with ninhydrin, i.e. all three substances present in *D* were adsorbed. The column was eluted with 0·1N-HCl but taurine, ethanolamine phosphate, and β all appeared as a single band. It was concluded that β must be strongly acidic. Later experiments using the method of Cohn & Carter (1950) suggest that separation on an anion-exchange column might be successful, but as other methods of separation had already been found this method was not developed.

Electrodialysis. A three-compartment electrodialysis cell was used, similar in construction to, but smaller (250 ml./cell) than that previously described (Campbell, Work & Mellanby, 1951). A sample fraction of *D* (250 ml.) containing 125 mg. N was placed in the centre compartment (pH 6·5), the anode compartment was filled with water and the pH adjusted to about 3 with dilute H_2SO_4, and the cathode compartment was brought to pH 9 with dilute NH_3. At 210 V. the maximum current was 1·5 amp., and after 3 hr. the current was 220 ma. Two-thirds of the contents of the anode and cathode compartments were withdrawn after the first 30 min. and replaced by distilled water. In three similar runs 434 mg. N (fraction *D*) were fractionated in this way. The final distribution of N in the three compartments was anode 45, centre 33 and cathode 22 %. A sample of the material from the centre compartment (fraction *E*) gave excellent two-dimensional chromatograms and showed the presence of taurine and the unknown substance β in approximately equal amounts. Practically all the ethanolamine phosphate was found in the anode compartment.

Paper columns. A paper column (3 × 55 cm.) was prepared with washed Solca Floc (100 g.) as described by Campbell, Work & Mellanby (1951). The acetone was replaced by a pyridine/amyl alcohol mixture made by mixing redistilled technical pyridine (1870 ml., b.p. 128–140°), amyl alcohol (1000 ml., pure technical), and water (940 ml.). A sample of fraction *E* (35 mg. N) was evaporated to dryness and mixed with the pyridine/amyl alcohol solvent (50 ml.). Not all the material dissolved and after some hours 41 mg. of crystals were collected and later identified as taurine. The solution of *E* (now about 30 mg. N) in pyridine/amyl alcohol was transferred to the column and the effluent collected in 5 ml. fractions on a fraction collector. The nature of the material, if any, in each fraction was determined by paper chromatography in pyridine/amyl alcohol. The taurine appeared in tubes 120–150, the unknown β in tubes 245–305, and a small quantity of ethanolamine phosphate in tubes 450–500.

Solvent was removed from the combined β fractions and samples of the residual oil were run on paper chromatograms in pyridine/amyl alcohol, phenol/NH_3, butanol/acetic acid and collidine. In each case the substance gave a single well defined spot after spraying with ninhydrin, and it was therefore assumed to be pure; nevertheless, all attempts at crystallization from various solvents failed.

Tubes 120–150 were worked up also and 19 mg. of crystalline taurine was isolated.

Characterization of substance β as glycerylphosphorylethanolamine

Despite our failure to crystallize substance β, we felt, from the chromatographic evidence, that we were justified in assuming we had isolated a pure specimen. This assumption was justified by subsequent experiment.

Acid hydrolysis. A sample was hydrolysed in a sealed tube at 110° for 16 hr. in 6N-HCl and the hydrolysate analysed by two-dimensional chromatography on paper, using phenol/NH_3 followed by collidine. There was no ninhydrin-positive spot in the position normally occupied by substance β, but instead a single spot approximately in the position normally associated with ethanolamine (Dent, 1948). A second sample of substance β was dissolved in 6N-HCl and left at room temperature for 2 hr. Acid was removed by leaving in a vacuum over KOH and the residue analysed by two-dimensional chromatography as before. Two spots were visible after spraying with ninhydrin, one corresponding to the original substance β and the second to ethanolamine.

Alkaline hydrolysis. A sample of substance β was hydrolysed with 0·5N-$Ba(OH)_2$ for 70 min. at 100°. The only

ninhydrin-positive substance produced was in the position of ethanolamine on a two-dimensional chromatogram.

Identification of ethanolamine derived from β. Although the ninhydrin-positive hydrolysis product from substance β followed ethanolamine in a two-dimensional chromatogram, this was not considered to be conclusive evidence as to its identity. Authentic samples of 3-amino- and 2-amino-propanol and of ethanolamine were mixed with samples of the hydrolysis product and run on paper in various solvents. No solvent could be found which could separate ethanolamine from our degradation product, but it could be separated from 3-amino- or from 2-amino-propanol in butanol/acetic acid.

As an additional check on the identity of the degradation product from substance β, a sample was oxidized by the method of Cooley, Ellis & Petrow (1950) by which ethanolamine is oxidized to glycine. The product of this oxidation in our case was also found to be glycine.

The identification of ethanolamine as a product of mild acid or alkaline hydrolysis suggested that we were dealing with an ethanolamine ester, and tests were accordingly made for an acid moiety involved in this ester linkage.

Absence of fatty acid from substance β. A sample of substance β was hydrolysed with HCl, and the hydrolysate tested for fatty acids by paper chromatography and by vapour phase chromatography (James & Martin, 1951). These experiments were kindly made for us by Dr A. J. P. Martin, F.R.S. No evidence could be found for the presence of a fatty acid.

Test for phosphorus. Two samples of substance β were run side by side on a paper sheet using propanol/NH_3 as solvent. One strip was sprayed with ninhydrin and the other was developed for P as described by Hanes & Isherwood (1949). The P reagent gave a strong blue spot in a position which corresponded exactly with the blue ninhydrin spot of the other chromatogram.

Ratio N : P. The absence of fatty acid and the presence of P, coupled with the instability towards alkali, suggested that we were dealing with a phosphate ester of ethanolamine and the N : P ratio was therefore determined by comparison with an authentic sample of ethanolamine phosphate. The NH_2 equivalent of substance β was determined by application of the ninhydrin colorimetric method of Harding & MacLean (1915) scaled down to 10 ml. total volume in place of the original 100 ml. Colour was measured in a Spekker absorptiometer. A standard curve was prepared from suitable dilutions of a solution of ethanolamine phosphate. P was also determined colorimetrically by the method of Allen (1940). A quantity of substance β containing 16 μg. P gave by the ninhydrin method a colour intensity equivalent to 9·0 μg. N and it was therefore concluded that the N : P atomic ratio was probably 1 : 1.

Glycerophosphoric acid, a degradation product of substance β. Samples of α- and β-glycerophosphate and of substance β were run on the same sheet of paper with propanol/NH_3 as solvent. The substances were located by the method of Hanes & Isherwood (1949). Substance β ran faster than either α- or β-glycerophosphate. A sample of substance β was then hydrolysed with 0·5 N-$Ba(OH)_2$ and after removal of Ba^{++} the product was again run on paper in parallel with α- and β-glycerophosphate. Colour development by the Hanes & Isherwood method revealed that substance β had been degraded to give α-glycerophosphate and β-glycerophosphate. This appearance of both glycerol esters is explained in the Discussion (p. 453). When substance β was hydrolysed with 5 N-HCl and chromatograms were run in the same way, a spot was found corresponding with inorganic phosphate as well as one in the glycerophosphate position.

The presence of a glycol grouping in substance β was confirmed by the method of Buchanan, Dekker & Long (1950). A sample of substance β was dissolved in water and a few drops of a dilute solution of $NaIO_4$ were added. The mixture was heated at 100° for 3 min. and water saturated with SO_2 added to destroy excess periodate. Finally, Schiff's reagent was added and the solution warmed again. A pink colour was obtained, indicating formation of an aldehyde from substance β.

Comparison of synthetic glycerylphosphorylethanolamine and substance β. A sample of synthetic glycerylphosphorylethanolamine (supplied by Prof. E. Baer) was mixed with an equal quantity of substance β, and attempts were made to separate the two compounds on paper chromatograms. The following solvent systems were tried, pyridine/amyl alcohol, butanol/acetic acid, collidine, phenol/NH_3. In no case was there any sign of separation; we therefore conclude that substance β is glycerylphosphorylethanolamine.

Glycerylphosphorylethanolamine in extracts of fresh liver: separation from citrulline

In order to ensure that glycerylphosphorylethanolamine was not an artifact produced as a breakdown product of some other substance, the following experiments were carried out.

Pig liver (220 g.) was placed on ice directly after its removal from the animal and homogenized in a Waring blender. The pH was adjusted to 5 and the homogenate (400 ml.) was poured into 2 l. of boiling distilled water. The

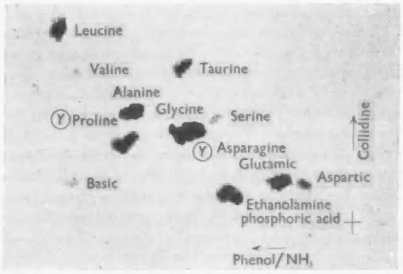


Fig. 1. Chromatogram of pig-liver extract. *Y* indicates the position of a yellow-coloured spot after development with ninhydrin which does not show on the photograph. 'Basic' refers to the presence of an unidentified basic amino-acid.

precipitated protein was filtered off and the filtrate concentrated to 670 ml. at 40–50°. The latter was dialysed against distilled water and the diffusate concentrated. A mercury precipitation was then carried out as previously described. After decomposition of the mercury salts a two-way chromatogram of the solution was run in phenol/NH_3 and collidine, and a satisfactory chromatogram obtained (Fig. 1). Similar chromatograms were obtained by treating the concentrated filtrate with ethanol and $CHCl_3$ as described by Awapara (1948). In the chromatograms produced by

both methods there was a large ninhydrin-positive spot in the position of citrulline on the diagram of spots shown by Dent (1948). This spot could be due to the presence of either citrulline or glycerylphosphorylethanolamine. Since citrulline forms a copper complex with copper carbonate, a decision may be reached as to the identity of the spot by re-running the chromatograms in phenol and collidine with copper carbonate on the chromatograms as described by Crumpler & Dent (1949). In this case only three spots developed after spraying with ninhydrin. These three spots (Fig. 2) were in the positions of taurine, glycerylphosphorylethanolamine, and ethanolamine phosphate (trace). There

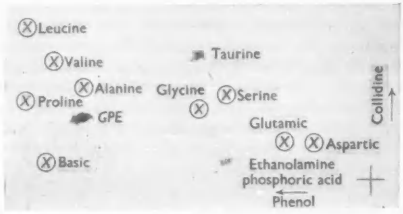


Fig. 2. Chromatogram of pig-liver extract treated with $CuCO_3.Cu(OH)_2$. *X* indicates the approximate position that the amino-acids shown in Fig. 1 would have occupied had the paper not been treated with $CuCO_3.Cu(OH)_2$. '*GPE*' refers to glycerylphosphorylethanolamine, and 'Basic' is as in Fig. 1.

is no doubt then that glycerylphosphorylethanolamine is present in fresh liver extracts. Without the use of copper carbonate it is very easy to confuse citrulline and glycerylphosphorylethanolamine as they fail to separate in pyridine/amyl alcohol, collidine, or phenol/NH_3. The two substances can, however, be separated in butanol/acetic acid in which citrulline runs faster than glycerylphosphorylethanolamine.

In another experiment a different fractionation procedure was used in which the initial heat treatment of the homogenate was avoided. The liver (20 g.) from a 5-week-old rabbit weighing 600 g. was homogenized in distilled water at 0° as previously described. The proteins were precipitated as described by Neuberg & Strauss (1945) by the addition of an equal volume of 21·2% (w/v) perchloric acid to the homogenate (100 ml.). The precipitate was filtered and the perchloric acid removed from the filtrate by the addition of 46 ml. of 50% (w/v) potassium acetate solution. The precipitate of potassium perchlorate was removed by filtration and 50 ml. of the filtrate concentrated to 4 ml. by freeze drying. The concentrate was added to 40 ml. of ethanol which was extracted with 120 ml. of $CHCl_3$ (Awapara, 1948). The aqueous layer was then treated with mercury as previously described. The decomposed mercury precipitate when run on two-dimensional chromatograms with and without copper carbonate as previously described, indicated the presence of glycerylphosphorylethanolamine. On the copper carbonate treated paper the spot due to taurine was very faint compared with that due to glycerylphosphorylethanolamine and of about the same intensity as that due to ethanolamine phosphoric acid. This confirms previous results with the usual fractionation procedure with Zeo-Karb 225, which showed that the amount of taurine in livers of young rabbits is very small.

Alternative method for isolation of taurine

As has been described, taurine may be isolated by the use of paper columns. However, a simpler method has been utilized which may be employed on a larger scale than is possible with paper columns.

The effluent from the Zeo-Karb 225 column (fraction *D*) was evaporated to dryness and then repeatedly extracted with hot pyridine to remove sugars (Malpress & Morrison, 1949). Very little ninhydrin-positive material is soluble in pyridine. The residue from the pyridine extraction was then dissolved in water and the solution was treated with phosphotungstic acid as described by Chibnall, Rees & Williams (1943). This phosphotungstic acid precipitate, after decomposition, was shown to have very little ninhydrin material, most of the latter remaining in the supernatant. The supernatant was then hydrolysed with 5 N-HCl in a sealed tube overnight, evaporated to dryness twice, and then passed through a column of the anion-exchange resin, Deacidite E (10 g.), to remove the remainder of the HCl. The effluent was then added to a column containing 0·4 g. of the cation-exchange resin, Dowex 50 (The Dow Chemical Co.), which had been activated with 5 N-HCl as described for the Zeo-Karb 225. The effluent from this column contained only one ninhydrin-positive substance, which appeared to be taurine. When the solution was concentrated and 4 vol. of ethanol were added to the hot solution, crystals appeared. The crystalline material, transferred to filter paper and chromatographed in amyl alcohol/pyridine, could not be distinguished from taurine. A sample of the crystalline material analysed satisfactorily as taurine. (Found: C, 20·0; H, 5·8; N, 10·9. Calc. for $C_2H_7O_3NS$: C, 19·2; H, 5·6; N, 11·2%.) The melting point of the crystals was 328°. The nitroprusside test was positive.

Altogether 22 mg. of crystalline taurine was obtained and it was calculated that from 1 kg. pig liver about 100 mg. could thus be obtained.

RESULTS AND DISCUSSION

The ninhydrin-positive water-soluble constituents of liver have been separated into two major fractions, those that are adsorbed on a cation-exchange resin, and those that are not. In pig-liver homogenates 57% of the water-soluble nitrogen was dialysable, of which 48% was adsorbed on the cation-exchange resin, Zeo-Karb 225. Zeo-Karb 225, rather than 215, was used in the present work. The former is a unifunctional sulphonated polystyrene resin which is lightly cross linked with divinylbenzene, whereas the latter is a sulphonated phenol-formaldehyde resin. The polystyrene resins have a greater molecular permeability than the phenol resins. Thus, Zeo-Karb 215 tends to adsorb irreversibly the aromatic amino-acids (Partridge, 1949), whereas the polystyrene resins (Zeo-Karb 225 and Dowex 50) do not. Since it was hoped in the present work to avoid irreversible adsorption of peptides present in liver extracts, polystyrene resins were obviously preferred.

This paper is chiefly concerned with the acidic

substances that are not adsorbed on a cation-exchange resin, but in pig liver the following substances have also been identified by chromatography: glutathione, aspartic acid, glutamic acid, ethanolamine, serine, glycine, glutamine, alanine, β-alanine, lysine, proline, valine, leucine, phenylalanine, and tyrosine (cf. Awapara, Landua & Fuerst, 1950*a*). We have not detected citrulline, although the methods used do not exclude its presence, and the same considerations apply to methionine. Ethanolamine is sometimes, but not always, present in the extracts and it is suspected that free ethanolamine may be a breakdown product of glycerylphosphorylethanolamine.

Only three ninhydrin-positive substances were consistently identified as being present in the effluent from the ion-exchange column and these were all precipitated as their mercury salts. Of these substances, glycerylphosphorylethanolamine and taurine represented the major part of the nitrogen from adult pig livers, whereas ethanolaminephosphoric acid only constituted a small proportion. In young rabbit livers, in which there is very little taurine, glycerylphosphorylethanolamine predominated.

Awapara, Landua & Fuerst (1950*b*) have shown that ethanolaminephosphoric acid occurs in many organs of the normal rat, and the results now obtained with pig liver confirm that it is not necessarily associated with tumour tissue as was at first thought by Outhouse (1937).

Awapara *et al.* (1950*a*) have reported the presence of taurine in rat liver, but did not isolate it. Our results suggest that in the particular pig liver concerned there was about 100 mg. of taurine/kg. so that the amount of taurine in the liver is quite considerable.

Although the method described here for the isolation of taurine from pig liver was fairly satisfactory, it is not regarded as the best possible method, and preliminary experiments with the strongly basic anion-exchange resin, Deacidite F (The Permutit Co. Ltd.) indicated that a simpler method for the isolation of taurine from fraction *A* of our present procedure could be devised.

In the isolation of glycerylphosphorylethanolamine from liver extracts the chief difficulty is in its separation from taurine. The two substances in many ways have very similar properties and the only way of separating the two which has so far been worked out is by the use of paper columns. This method, however, suffers from the fact that it can deal only with comparatively small quantities. In our isolation of glycerylphosphorylethanolamine, electrodialysis was used, but although this procedure removed a certain amount of interfering material, and most of the ethanolaminephosphoric acid, the loss of nitrogen (only 25 % remained in the centre compartment) was considerable and there is little doubt that there was a loss of glycerylphosphorylethanolamine in this step.

Glycerylphosphorylethanolamine has been found not only in pig liver but also in the liver of rabbits and in a foetal sheep liver by us, and in low concentration in rat liver (Walker, 1951). It has also been identified in this Institute in an extract of brewer's yeast. Dr Walker has also detected it in ox spleen, kidney, brain, and liver. The possibility that glycerylphosphorylethanolamine is split from some larger labile molecule, e.g. phospholipin, during the course of isolation, cannot be completely excluded. However, glycerylphosphorylethanolamine was detected in liver extracts when the protein was removed by cold perchloric acid (Neuberg & Strauss, 1945) and also when acid was excluded and the protein precipitated by heat. It is very probable, therefore, that glycerylphosphorylethanolamine occurs as such in the cell.

The identification of glycerylphosphorylethanolamine in the tissues of various animals is, so far as is known, the first report of the occurrence of this substance free in cells. Feulgen & Bersin (1939) obtained it in crystalline form as the hydrolysis product of muscle plasmalogens, and recently Thannhauser, Boncoddo & Schmidt (1951) obtained it from a brain plasmalogen. In the latter case the α isomer exclusively was obtained. Since the sample of glycerylphosphorylethanolamine obtained in the present work reacted with periodate under conditions in which α- but not β-glycerophosphates react, it is certain that some of the α isomer was present. However, chromatography in propanol-ammonia, in which solvent it is possible to separate the α- and β-glycerophosphates, indicated that on alkaline hydrolysis both glycerophosphates were produced. Baer & Kates (1948*b*) have already shown that on hydrolysis of glycerylphosphorylcholine an equilibrium mixture of the α- and β-glycerophosphates is produced. Thus the production of β-glycerophosphate on hydrolysis of glycerylphosphorylethanolamine does not necessarily indicate the presence of β-glycerylphosphorylethanolamine in the sample.

That glycerylphosphorylethanolamine has not previously been identified in tissue extracts would seem to be due to its instability and its association with citrulline in two-dimensional chromatograms. The two substances run identically in the solvents normally used for two-dimensional chromatography, namely phenol/NH_3 and collidine, and also in amyl alcohol/pyridine. However, the two substances are separated by a fourth solvent, namely butanol/acetic acid. The chromatographic similarity between two substances with such different structures is rather surprising and suggests a note of caution in too ready an acceptance of chromato-

graphic evidence alone in the identification of unknown substances.

No experiments have yet been carried out to determine the biological significance of glycerylphosphorylethanolamine. It would be surprising if this phosphate ester were not of biological significance since it is present in comparatively high concentrations in the livers of a variety of animals and apparently also in other tissues. Fig. 2 is typical of the chromatograms obtained from the liver extracts of various animals.

SUMMARY

1. The nitrogenous water-soluble constituents of liver have been separated into two groups by use of an ion-exchange resin, Zeo-Karb 225.

2. The group of compounds not adsorbed on Zeo-Karb contained three substances giving coloured derivatives with ninhydrin.

3. Two of these substances were identified as taurine and ethanolaminephosphoric acid.

4. Two methods were devised for the isolation of crystalline taurine from pig liver.

5. The third 'ninhydrin-positive' substance was isolated from pig liver and, by degradative methods and by comparison with a synthetic specimen, it was shown to be glycerylphosphorylethanolamine.

6. Evidence was obtained that glycerylphosphorylethanolamine is a normal constituent of liver and not an artifact produced from phospholipins.

7. Glycerylphosphorylethanolamine was found in rabbit liver and foetal sheep liver as well as in pig liver. It is also found in other types of tissue.

Our thanks are due to Dr J. G. Buchanan for a gift of samples of 3-amino- and 2-amino-propanol, and advice as to their chromatographic behaviour; to Dr V. Petrow for details of the conversion of ethanolamine to glycine on a micro scale; to Dr K. W. Pepper, of the Chemical Research Laboratory, Teddington, for helpful discussions concerning the properties of sulphonated polystyrene resins; to Prof. E. C. Amoroso for the foetal lamb liver; and to Prof. E. Baer, of the University of Toronto, who very kindly sent us a sample of glycerylphosphorylethanolamine prior to the publication of his work, and when only a few mg. had been prepared. Finally, we should like to thank Mr S. Krol for his skilled technical assistance throughout the course of this work.

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The Assay of Vitamin B_{12}. Relationship between Growth Response of *Lactobacillus leichmanii* 313 in Tubes and Diffusion of Oxygen into the Medium

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The microbiological tube assay for vitamin B_{12} with micro-aerophilic Lactobacilli as test organisms requires careful control of E_h in the assay medium if uniform responses to the vitamin are to be obtained. The presence of reducing substances and degree of aeration of the test medium are the main factors in determining this E_h. Apart from substances that may be added in the dilutions of the test sample, the main source of reducing material is the interaction of constituents of the medium during sterilization in the autoclave. A rigorously standardized autoclaving procedure, as described by Shaw (1948), is the best method of controlling this variable. The effects of aeration and reducing substances on the growth response of *Lactobacillus lactis* Dorner to vitamin B_{12} have been described by Kocher (1949) and by Koditschek, Hendlin & Woodruff (1949).

When attempting to adapt our previously described tube assay method (Lees, Emery & Tootill, 1951) to an agar plate technique for the examination of paper strip chromatograms, we found incorporation of a reducing agent in the medium necessary for growth response to vitamin B_{12} in the relatively thin agar layer. Because the reducing agent is indispensable in the plate assay and because of an observation relating the depression of response in the turbidimetric tube assay with abnormally wide tubes, we have examined in more detail the effects of the diffusion of oxygen into the medium on the growth response of *Lb. leichmannii* 313 to vitamin B_{12} and have tried to assess their magnitude.

EXPERIMENTAL

The conditions of assay employed were those described by Lees *et al.* (1951). Briefly, a sterile medium adequate for the nutritional requirements of the test organism, but free of vitamin B_{12}, is inoculated with a washed suspension of the test organism; after incubation graded responses are obtained according to the amounts of vitamin B_{12} added to the tubes. The response is determined by reading the turbidity directly in the Spekker photoelectric absorptiometer on a logarithmic scale, which gives a reading approximately proportional to the amount of growth. To obtain the most precise measure of the magnitude of the effects, these conditions were varied so as to involve bulk inoculation of the test organism and vitamin B_{12}, whenever the design of the experiment permitted it.

In view of the importance of autoclave timing when the basal tube medium is being sterilized, we give below the essential details of our procedure. The metal racks containing spaced tubes were placed in the autoclave and subjected to free steaming (no rise from atmospheric pressure) for 5 min. The valves were closed and the pressure was allowed to reach 15 lb./sq.in. during an interval of 1·5 min. The steam was then turned off and the pressure allowed to equilibrate with the atmosphere in 1·5 min. by adjustment of the exhaust valves. The racks were immediately removed and cooled in water.

RESULTS

To verify our observations relating the depression of growth response of the Lactobacillus in certain tubes to their abnormal width, we first examined the response of the test organism to vitamin B_{12} in a series of stock tubes of increasing internal diameter, each tube containing a constant volume of medium.

It was found that with doses of vitamin B_{12} below 0·23 mμg./tube, some tubes showed no growth whatsoever, whereas with doses in excess of 1·20 mμg./tube, many tubes showed no gradation in turbidity against further increase of dose since maximum growth had already been obtained.

Accordingly, the responses for doses in the range 0·23–1·20 mμg./tube were analysed and are shown in Table 1 with the accompanying analysis.

The analysis shows that a highly significant linear component of regression of growth on surface area exists, with a smaller, but still highly significant, deviation from linear regression.

It was considered that this deviation from the linear component was most probably due to the effect of depth of medium on the growth response, although the deviation could also be explained by the existence of a curved regression of response on surface area. An experiment with uniform tubes, each containing the same volume of medium but having varying surface areas exposed to the atmosphere, served to show that a linear regression between growth and surface area was sufficient to account for the data when depth was maintained constant. To obtain the varying surface areas a series of paraffin wax annuli of known internal diameters were floated on the surfaces of the medium in the tubes, the periphery of each annulus being sealed to the inner wall of the assay tube by means of a small flame.

Table 1. *Growth response related to area of tube exposed to air*

(Growth response is given as turbidity readings using the Spekker absorptiometer.)

Tube internal cross-sectional area (sq.cm.)	Doses of vitamin B_{12} (mμg./tube) 0·23	0·35	0·53	0·80	1·20
	Growth response				
1·54	0·78	1·13	1·28	1·40	1·56
	0·80	1·11	1·19	1·42	1·56
2·14	0·62	0·98	1·10	1·37	1·48
	0·74	1·03	1·12	1·36	1·45
4·30	0·06	0·11	0·24	0·46	0·56
	0·06	0·07	0·22	0·40	0·77

Analysis of variance

Source of variation	Degrees of freedom	Squares	Mean square	Ratio	Probability
Regression on tube surface area	1	5·134,12	5·134,12	1989·97	$P<0·001$
Deviation from area regression	1	0·064,84	0·064,84	25·13	$P<0·001$
Differential response of doses on tubes	8	0·086,04	0·010,76	4·17	$P<0·010$
Doses	4	1·892,42	0·473,11	—	—
Error	15	0·038,65	0·002,58	—	—
Total	29	7·216,07	—	—	—

Table 2. *The effect of paraffin wax annuli on growth response*

Diameter of hole (mm.) ...	16	11	9	8	7	6	5	4	0
Duplicate responses	0·70	0·84	0·78	0·93	0·90	0·97	0·99	0·86	0·98
	0·64	0·95	1·03	0·92	1·03	0·91	1·05	1·00	1·09
Mean response	0·670	0·895	0·905	0·925	0·965	0·940	1·020	0·930	1·035

Analysis of variance

Source	Degrees of freedom	Squares	Mean square	Ratio	Probability
Regression on area of medium exposed	1	0·1612	0·1612	21·65	$P\approx 0·001$
Deviation	7	0·0171	0·00244	0·33	Not significant
Error	9	0·0670	0·00745	—	—
Total	17	0·2453	—	—	—

Table 2 presents the results and analysis of this experiment, showing a linear regression of growth response on the area of the medium exposed to the air, with a deviation from this regression well below significance.

These results made us reasonably confident that the effects were due to atmospheric oxygen and, since the assay medium is stationary during growth of the test organism, it was assumed that oxygen entered the medium by solution at the surface and reached the lower level by diffusion only. It was further assumed that inhibition of growth due to dissolved oxygen would, with increasing depth, decrease asymptotically to a finite value or zero, and that the growth in any given lamina of medium would thus be related to its depth below the surface of the medium. The turbidity of an infinitesimal lamina being impossible to measure, the contents of the tubes were dispersed by shaking before turbidity determinations, to give a measure of the average turbidity throughout the medium. It was thought that the limiting value of inhibition would be attained at not too great a depth below the surface and that the average turbidity we measured could therefore be considered as a dilution of the maximum growth attainable below this depth with the subnormal growth obtained at lesser depths. Thus it was expected that the average growth throughout the medium would be inversely related to the depth, at least beyond the point at which the limiting value was obtained. Accordingly an experiment was set up with varying depths of medium in tubes selected for uniform internal diameter. The statistical analysis of the results in Table 3 shows that the inverse relationship was applicable to our experiment in depths of medium corresponding to 8 ml. and upwards in 6×1 in. assay tubes.

The assumption was made that the inhibition, I, of growth in an infinitesimal lamina at a depth

Table 3. *Growth response of test organism according to the depth of the assay medium*

Volume in 1 in. tubes (ml.) ...	2	4	6	8	10	12	14	16	18	20
Replicate responses	0·50	0·58	0·66	0·79	0·91	1·01	1·07	1·14	1·12	1·16
	0·50	0·59	0·70	0·83	0·91	1·01	1·07	1·15	1·19	1·15
	0·52	0·58	0·70	0·80	0·93	1·01	1·10	1·10	1·15	1·17
Mean responses	0·507	0·583	0·687	0·807	0·917	1·010	1·080	1·130	1·153	1·160

Analysis for reciprocal relation between mean response and depth of medium from 8 ml. upwards

Source	Degrees of freedom	Squares	Mean square	Ratio	Probability
Regression	1	0·31996	0·31996	772·85	$P<0·001$
Deviation	5	0·00231	0·000462	1·11	Not significant
Error	14	0·00580	0·000414	—	—
Total	20	0·32807	—	—	—

corresponding to a volume v in the tube would be given by the formula

$$I = A \exp(-\tfrac{1}{2}k^2v^2).$$

It was observed that this formula is similar to one given by Cooper & Woodman (1946) for the diffusion of antiseptics through agar gels, where volume has replaced distance as a variable, since in tubes of constant diameter volume is proportional to depth. Further, since a constant time of incubation was employed for all experiments, time, which appears as a variable in Cooper & Woodman's formula, has been included in the constant k. In effect, the equation assumes that the inhibition is simply related to the oxygen concentration obtained by diffusion.

If g represents growth in a lamina of medium corresponding to volume v

g_o growth at the surface of the medium, i.e., in conditions of oxygen saturation

and g_∞ growth at 'infinite' depth, i.e., in complete absence of diffused oxygen, then

$$g = g_\infty - I,$$

$$g = g_\infty - A \exp(-\tfrac{1}{2}k^2v^2),$$

and

$$\frac{g_\infty - g}{g_\infty - g_o} = \exp(-\tfrac{1}{2}k^2v^2).$$

The measurements are made on the average growth obtained throughout the tube, as described above; they can be obtained from the formula by summing the growth in all laminae and dividing the result by the volume concerned. Thus if $\bar{g}$ represents the average growth obtained in a volume v, then

$$\frac{g_\infty - \bar{g}}{g_\infty - g_o} = \frac{1}{kv} \exp(-\tfrac{1}{2}k^2v^2)\, \mathrm{d}(kv).$$

Define $\phi(X)$ by

$$\phi(X) = \int_0^x \mathrm{e}^{-\frac{1}{2}x^2}\, \mathrm{d}x.$$

Then

$$\frac{g_\infty - \bar{g}}{g_\infty - g_o} = \frac{1}{kv}\, \phi(kv),$$

and thus $\bar{g}$ is linearly related to $\frac{1}{v}\phi(kv)$, whose value for any specified volume is known when k is known. The value of k may be chosen so as to make the regression of $\bar{g}$ on $\frac{1}{v}\phi(kv)$ as nearly linear as possible. (Values of ϕ for any value of the product kv may be read very easily from tables of the normal probability integral.)

An approximate value for k was determined graphically and the values of $\frac{1}{v}(\phi kv)$ for the volumes used in the experiment were calculated for graded values of k in the neighbourhood of the approximate value previously obtained. The truth of the above hypothesis relating aeration of test medium to inhibition of the test organism implies linear regression between the experimentally observed results and the values of $\frac{1}{v}\phi(kv)$ for the maximum likelihood value of k.

Thus the sum of squares attributable to this linear regression was calculated for the graded values of k and the maximum value determined by interpolation; this maximum value corresponds to a minimum mean square value for the deviation from the regression, so determining the maximum likelihood value of k.

The tabulated values of the above functions and the corresponding derived results (based upon the maximum likelihood value of $k = 0·222,54$) are given in Table 4.

The analysis in Table 5 shows this deviation to be non-significant, and the formula therefore accounts for all the relevant variation experienced and is shown to be statistically valid.

The value of k determined in the above manner enabled the constants of the linear regression between the experimental results and $\frac{1}{v}\phi v$ to be evaluated, thus giving values for g_0 and g_∞. The values of g and $\bar{g}$ are shown graphically in Fig. 1, the experimental results being superimposed as circles.

Table 4. *Experimental results shown with maximum likelihood values of theoretical formula*

v = volume in tubes	$\phi' kv = \exp(-\frac{1}{2}k^2v^2)$	g = theoretical growth in lamina of medium corresponding to volume v	$\frac{1}{kv}\phi(kv) = \frac{1}{kv}\int_0^{kv} \exp(-\frac{1}{2}k^2v^2)\,d(kv)$	$\bar{g}$ = calculated responses	Mean experimental responses
0	1·000,00	0·467	1·000,00	0·467	—
2	0·905,74	0·561	0·967,95	0·499	0·507
4	0·672,88	0·794	0·882,26	0·584	0·583
6	0·410,07	1·056	0·768,00	0·699	0·687
8	0·204,99	1·261	0·561,16	0·815	0·807
10	0·084,06	1·382	0·548,52	0·918	0·917
12	0·028,28	1·438	0·465,77	1·001	1·010
14	0·007,80	1·458	0·401,54	1·065	1·080
16	0·001,77	1·464	0·351,86	1·114	1·130
18	0·000,33	1·466	0·312,86	1·153	1·153
20	0·000,05	1·466	0·281,59	1·185	1·160

Table 5. *Analysis to test the statistical validity of theoretically derived formula*

Source	Degrees of freedom	Squares	Mean square	Ratio	Probability
Function	2	1·611,310	0·805,655	2237·93	$P<0·001$
Deviation	7	0·004,357	0·000,622	1·73	Not significant
Error	20	0·007,200	0·000,360	—	—
Total	29	1·622,867	—	—	—

Further experiments were conducted, designed to examine the effect of various levels of vitamin B_{12} on the shape of the inhibition curve shown by the lower line in Fig. 1. The relationship between the mean growth response and the volume of medium in the tube for three levels of vitamin B_{12} is shown in Fig. 2. The experiment could have been improved by using a logarithmically spaced series of doses.

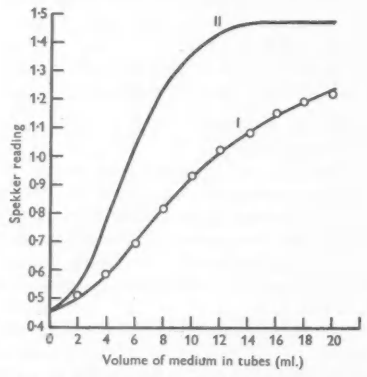

Fig. 1. Experimental turbidities (○) obtained at varying depths superimposed upon the theoretically derived relationship between depth of medium and turbidity of growth: Curve I, $\bar{g} = A - \frac{B}{kv}\int_0^{kv} \exp(-\frac{1}{2}k^2v^2)\,d(kv)$. Assumed curve showing the turbidity in individual laminae of medium at varying depths: Curve II, $g = A - B\exp(-\frac{1}{2}k^2v^2)$.

DISCUSSION

The curves in Fig. 2 show the relationship between volume of medium and density of growth obtained at the end of incubation. A simple interpretation of this gradation of growth in the light of oxygen diffusion is not possible, because growth takes place over a period of hours and oxygen concentration at any given depth varies with time during this period. The growth of the organism in any horizontal

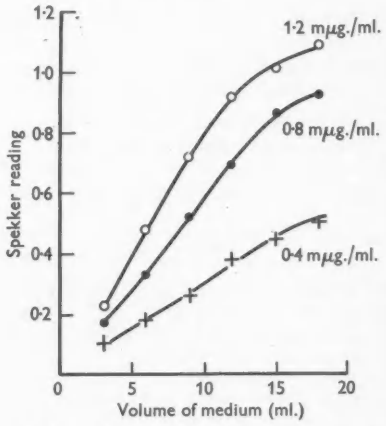

Fig. 2. Relationship between growth of *Lactobacillus leichmannii* 313 at varying depths for three doses of vitamin B_{12}.

lamina is taking place in the presence of oxygen concentration increasing with time, but the curves of oxygen concentration with respect to depth at all times are similar in shape, thus supporting an interpretation of the terminal incubation conditions relating inhibition of growth to oxygen diffusion from the atmosphere. The apparent inhibition of the test organism noted may be accentuated by a gradual settling of the organisms during incubation.

To minimize the interference from oxygen in the test medium in routine tube assay of vitamin B_{12}, it is necessary to use tubes carefully selected for uniform internal diameter and to ensure that the conditions of aeration of the medium, after autoclaving, are as nearly as possible identical for the standard test sample assay tubes. This can be conveniently achieved by allowing the autoclaved tubes to attain atmospheric equilibrium before inoculation or by allowing only an insignificant interval of time to elapse between inoculation of standard and test sample assay tubes, a procedure only possible when few unknowns are being assayed against one standard preparation.

The accuracy of any microbiological assay depends to a certain extent upon the slope of the dose-response curve. Examination of Fig. 2 shows that this slope, i.e. the difference in absorptiometer reading for a standard dose increment, increases rapidly until a volume of 15–20 ml./tube is used, when no further practical advantage is gained by the use of larger volumes. With this information, we improved our assay method by using narrower tubes with our original volume of 10 ml., thus increasing the depth of the medium, which achieves the same end as an increase in its volume. We recommend that workers performing tube assays employing micro-aerophilic test organisms should examine the slope of the dose response curve with respect to the depth of medium in the assay tubes, obtaining such a relationship as is shown in Fig. 2, whence the most suitable depth of medium can easily be determined.

In determining the most suitable volume of medium for microbiological tube assays employing micro-aerophilic test organisms, it should be borne in mind that this may be affected by constituents of the medium. Schweigert, Guthneck & Schneid (1950), employing medium volumes of 2 ml./tube, found that the replacement of cystine with cysteine greatly improved the growth of *Lb. leichmannii* 313; it is clear that in this small volume the reducing properties of cysteine are effective in overcoming the inhibition due to the highly aerobic medium. This interpretation is supported by the fact that for optimum growth of *Leuconostoc mesenteroides* much lower levels of cysteine and cystine are required. Cystine, when autoclaved with hydrolysed casein, is partially converted to a substance with growth-promoting properties much exceeding those of the original cystine added to the medium. Cysteine added aseptically to the medium after autoclaving also yields good growth, suggesting that cystine is, to a certain extent, converted to cysteine, whose reducing properties are the important factor in promoting good growth of lactobacilli.

SUMMARY

1. The relationship between the growth response of *Lactobacillus leichmannii* 313 to vitamin B_{12} and the oxygen content of the assay medium has been investigated.

2. A simple theory of inhibition of the test organism on the basis of diffusion of atmospheric oxygen has been postulated and found to account satisfactorily for the experimental observations.

3. It is recommended that the optimum conditions for microbiological assays with micro-aerophilic test organisms should be established after examining the growth response of the organism along lines similar to those described in the paper.

We wish to acknowledge the valuable technical assistance given in this investigation by Miss M. Hawksley and Mr D. G. Goodinson.

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Studies in Immunochemistry

10. THE ISOLATION AND PROPERTIES OF LEWIS (Le^a) HUMAN BLOOD-GROUP SUBSTANCE

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During the last few years a considerable body of evidence has accumulated which indicates that mucoids, or complexes consisting of polysaccharides and amino-acids, are responsible for certain of the serological blood-group characters of human erythrocytes, tissue fluids and secretions. There are as yet, however, many blood-group properties which, although firmly established on serological and genetical grounds, remain, nevertheless, unassociated with any chemically defined substance.

Mourant (1946) discovered a new human blood-group character called Lewis and found that the red blood cells of about 25% of English people are agglutinated by anti-Lewis sera. The agglutination is independent of the other known blood-group characters, such as those shown by ABO, MN, *Rhesus*, P, Lutheran, and Kell systems. A further Lewis character was subsequently identified by Andresen (1948) and the two Lewis genes responsible for the serological and genetical behaviour observed up to that time were designated Le^a and Le^b, the corresponding antibodies as anti-Le^a and anti-Le^b and the gene products as Le^a substance and Le^b substance (Andresen, Callender, Fisher, Grubb, Morgan, Mourant, Pickles & Race, 1949).

The examination of a large number of bloods and salivas by Grubb (1948) and by Grubb & Morgan (1949) showed that the secretions of individuals who are 'Lewis positive', i.e. persons whose erythrocytes are agglutinated with anti-Le^a serum, do not contain the A, B and H substances which are to be expected according to the ABO blood group of the individual. It was also observed that the secretions from Lewis-positive persons always contain Le^a substance (cf. Brendemoen, 1949) and that, therefore, this gene product would be most easily isolated from the tissue fluids and secretions of individuals with Lewis-positive erythrocytes.

The isolation and characterization of the mucoid which is responsible for the Le^a character of human erythrocytes and secretions forms the subject matter of the present paper.

EXPERIMENTAL

The methods employed for the isolation and purification of the Lewis Le^a substance from ovarian cyst fluids were similar to those described earlier for the group A and H substances (King & Morgan, 1944; Morgan & Waddell, 1945; Aminoff, Morgan & Watkins, 1950). The importance of using gentle procedures for the isolation of blood-group substances is now well known, and accordingly methods designed to bring about a minimum of change in these labile complexes have been employed.

Materials and methods

General. In earlier experiments ovarian cyst fluids were selected which contained the Le^a substance, irrespective of whether the more common group factors A, B, H and Lewis Le^b were also present. The discovery that Lewis-positive persons secreted Le^a substance without the other group substances indicated that for the isolation of the Le^a substance the cyst fluids obtained from Lewis-positive persons were the materials of choice.

Determination of serological activity. The iso-agglutination inhibition tests used throughout the investigation were as described by Grubb & Morgan (1949) and employed an anti-Le^a serum, 'Hughes', which was obtained from an individual with the following group characters: A_2, Le(a −), secretor of A and H substances, CDe/CDe, Kell negative, Lu(a −). The test cells used were always obtained from the same person, were group O Lewis-positive and were agglutinated to a titre of 16 by the serum 'Hughes'. A 'standard' preparation of Le^a substance was included in all tests, which were read after standing for 2–3 hr. at 14–16°. Tests used to determine the presence or absence of A and B substances in the cyst fluids were performed as described by Morgan & King (1943). Cyst fluids derived from persons belonging to group O were examined for H substance according to the procedure of Morgan & Waddell (1945).

Total nitrogen. N content was determined by the Kjeldahl method, using the apparatus of Markham (1942) and the bromocresol green-methyl red indicator of Ma & Zuazaga (1942). Occasionally the N content of the final preparations was checked by the Dumas micromethod (Pregl, see Grant, 1945).

Acetyl content. This was determined by the method of Kuhn & Roth (1933) as described by Grant (1945) (see Aminoff *et al.* 1950). A heating period of 6 hr. is necessary to obtain the maximum acetyl value for the Le^a substance.

Estimation of carbohydrate components. The total fucose was determined by the method of Dische & Shettles (1948) employing a Hilger Uvispek spectrophotometer. The fucose content was calculated on the basis of the difference in the extinctions at 400 and 430 mμ. The acid-labile fucose was determined, after hydrolysis of the Le^a substance with N-acetic acid at 100° for 48 hr., by measuring the acetaldehyde liberated on treatment of the hydrolysis products with

periodate (Aminoff *et al.* 1950). The method was slightly modified by carrying out the oxidation in a stronger phosphate buffer (0·4 M) at pH 7·5 instead of pH 7·0 as used earlier. Under these conditions 95–100% of the theoretical amount of acetaldehyde was recovered from fucose.

Reducing power was determined as glucose by the copper-reduction method of Somogyi (1937).

N-Acetylhexosamine was determined according to Morgan & Elson (1934), using a photoelectric colorimeter. Acetylglucosamine concentrations between 10 and 40 μg./ml. were employed as standards.

Glucosamine and chondrosamine were assayed by a modification of the method described by Elson & Morgan (1933), using glucosamine hydrochloride concentrations of 10–40 μg. as standards, a photoelectric colorimeter and a green light filter (maximum transmission, 540 mμ.).

Chromatographic methods. Amino-acid analyses were carried out by the two-dimensional paper-partition chromatographic technique of Consden, Gordon & Martin (1944), using as solvents *s*-collidine and phenol, and, as supporting medium, Whatman no. 1 paper. Carbohydrate-containing areas were revealed by spraying the paper with $AgNO_3$-NH_3 (Partridge, 1946) or with aniline hydrogen phthalate (Partridge, 1949). The areas on the paper which contained amino sugars were indicated by treatment with alkaline acetylacetone followed by *p*-dimethylaminobenzaldehyde-HCl in *n*-butanol (Partridge, 1948).

α-Amino N and α-amino-acid N. α-Amino N was determined by measuring the N_2 liberated on treatment with HNO_2 in the Van Slyke-Neill manometric apparatus (Peters & Van Slyke, 1932). α-Amino-acid N was determined by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) at pH 2·5 and a reaction time of 8 min.

The isolation of the Le^a *substance*

The methods used for the isolation and purification of the Le^a substance were very similar to those described for the human blood-group A substance (Aminoff *et al.* 1950).

A typical procedure, applied to cyst fluid 'UN', was as follows: the cyst fluid (2·5 l.) was dried from the frozen state, yielding 83 g. of material. Previous experience had shown that extraction of the dried cyst material with 90% phenol was usually the most effective first step in the isolation of the specific substance, and the results of a preliminary experiment in this instance also showed that the active material was concentrated largely in the phenol-insoluble residue, whereas most of the contaminating protein dissolved in the phenol. The whole of the dried cyst material was therefore treated repeatedly with 90% (w/v) phenol, and a phenol-insoluble material (10·6 g.; N, 7·4%) was obtained. This material was only partially soluble in water. The water-insoluble residue (5·1 g.; N, 9·9%) dissolved in dilute NaOH at 0°, but the resulting solution showed little Le^a activity. The main water-soluble component was thoroughly dialysed and dried from the frozen state. The material (5·3 g.; $[\alpha]_{5461}$ – 35°; N, 6·0%) was highly active in iso-agglutination inhibition tests against Le^a agglutinin and a part of this material was set aside and subsequently employed as the laboratory 'standard' Le^a substance.

The N content of the water-soluble material indicated that the preparation was substantially uncontaminated with free protein, but nevertheless the material was put through the deproteinization technique described by Sevag (1934). The results of preliminary experiments indicated that the deproteinization proceeded most effectively at pH 4·6. The whole of the main preparation of Le^a substance was therefore dissolved to yield a 0·5% solution in 0·1 N-Na acetate buffer, pH 4·6, and was vigorously stirred with an equal volume of a 1:1 mixture of $CHCl_3$ and amyl alcohol in 250 ml. centrifuge pots for 20–30 min. The two liquid phases were then separated by centrifugation, the supernatant fluid was siphoned off and the denatured protein in the organic solvent layer washed with an equal volume of fresh buffer. The aqueous supernatant fluid and the buffer washings were pooled and treated again at pH 4·6 with the organic solvent mixture. The aqueous supernatant fluid was given a third treatment with $CHCl_3$-amyl alcohol, dialysed and dried, from the frozen state. The product (3·1 g.; $[\alpha]_{5461}$ – 50°; N, 5·25%) showed high Le^a activity. The insoluble material which remained suspended in the organic phase and at the solvent interface was collected by pouring the suspension into excess ethanol and separating the solid by centrifugation. An appreciable amount of Le^a substance separated with the denatured protein, but this material could be largely recovered by repeating the Sevag deproteinization procedure. The substance separating at the solvent interface yielded a final product (2·0 g.; $[\alpha]_{5461}$ – 46°; N, 7·9%) which showed about half the serological activity of the water-soluble material.

The highly active material obtained after deproteinization by Sevag's technique was separated into six fractions by the addition of ethanol to a 1·5% aqueous solution of the substance. The fractionation was carried out at 0° in the presence of 1% of potassium acetate. After the adjustment of the ethanol concentration to a new level the suspension was allowed to stand for 30–40 min. at 0° before the material was collected by centrifugation. The analytical figures obtained for the six fractions (Table 1) suggest that the original material was not grossly inhomogeneous chemically. All fractions were equally active serologically when expressed in terms of the standard Le^a substance. Some 80% of the total material was thrown out of solution over the range 50–54% (v/v) ethanol, but electrophoretic examination at pH 8·0 in

Table 1. *The fractionation of* Le^a *substance from aqueous solution by the addition of ethanol at* 0°

Fraction no.	Precipitation level (% (v/v) ethanol)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)
1	40–44	0·01	—*	5·5
2	44–47	0·06	—	5·2
3	47–50	0·17	—	5·4
4	50–54	2·19	– 38°	5·3
5	54–57	0·13	– 28°	5·2
6	57–Excess	0·18	– 36°	5·4

* Too opalescent for accurate observation.

phosphate (I, 0·2) revealed the presence of two components in almost equal amounts. The low mobilities of both substances and their fairly rapid diffusion under the conditions of electrophoresis rendered any attempt at electrophoretic separation impracticable. The possibility of separating the mixture by fractional centrifugation was considered, but examination in the ultracentrifuge showed that the material sedimented as a single component (Fig. 1). The area of the peak given by the diagonal schlieren optical system accounted for almost the whole of the material and suggested that the components of the mixture were of similar particle weights or possessed compensating shapes.

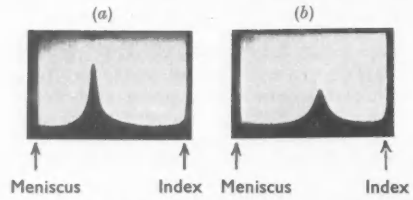

Fig. 1. Sedimentation diagrams of a 1% solution of Le^a substance in phosphate (pH 8·0, $I = 0·2$): (*a*) 50 min. and (*b*) 80 min. after reaching full speed (270,000 *g*).

In view of the success which had attended the isolation of the group A substance following treatment of the native cyst fluid material with trichloroacetic acid at 0°, the possibility of applying this method to the purification of Le^a substance was examined. The possible degradation of the Le^a complex with loss of specific serological properties had to be considered, however, and a preliminary experiment with a side fraction was therefore first undertaken. Without giving details of the analytical figures of the materials obtained on subsequent fractionation of material rendered soluble in phenol by treatment with trichloroacetic acid, it may be stated that an appreciable increase in specific serological activity was shown by the material precipitated from solution in 90% phenol by the addition of ethanol up to 10% (v/v) concentration. An electrophoretic examination of this material at pH 8·0 showed the presence of a fast-moving substance, presumably protein, which accounted for about 10% of the preparation, and two other components of low mobility which from the areas of their peaks in the diagonal schlieren diagrams were present to the extent of approximately 70 and 20%. Electrophoretic analysis of the Le^a substance prior to the application of the trichloroacetic acid-phenol technique showed that the material contained these two components in nearly equal amounts. It seemed probable, therefore, that this treatment had preferentially rendered phenol-soluble one of the two major components of the inhomogeneous specimen of Le^a substance used.

The remaining material from cyst fluid 'UN' which was insoluble in 90% phenol was treated with trichloroacetic acid and subsequently fractionated from phenol solution by ethanol. An electrophoretically homogeneous specimen of Le^a substance was not isolated; the analytical figures for the most active materials obtained from this cyst fluid fell within the range: N, 5·4–5·6%. $[\alpha]_{5461}$ −28 to −36°.

A second cyst fluid, no. 123, obtained from a Lewis-positive person belonging to group A_2, was dried from the frozen state. The material weighed 58 g. and possessed 6% of the serological activity of the 'standard' Le^a substance. Extraction of the material with 90% liquid phenol yielded 6·5 g. of phenol-insoluble residue which was found to contain almost the whole of the Le^a specific substance. This material was only partially soluble in water; the aqueous extract after dialysis gave 1·6 g. of water-soluble material ($[\alpha]_{5461}$ −40°; N, 5·7%) which was serologically as active as the 'standard' preparation. The material was deproteinized by the Sevag procedure as described above, and gave a product (1·2 g.; $[\alpha]_{5461}$ −40°; N, 4·8%) which showed no enhancement of serological activity.

It was observed that the active material, although insoluble in 90% phenol, was largely soluble in a solution of 90% phenol which contained 20% (v/v) ethylene glycol, and could be recovered from this solvent mixture by addition of excess phenol. This solution and precipitation procedure was repeated twice on the active material with the object of removing any non-dialysable peptide of low molecular weight which was not removed by the Sevag procedure. The analytical figures of the product recovered, however, were identical with those of the starting material.

About 1 g. of the partially purified Le^a substance was dissolved in phenol-glycol mixture and was recovered by the gradual addition of increasing quantities of ethanol. The results of the fractionation are given in Table 2. The active substance was precipitated over a wide range of ethanol concentration, but no material was obtained which differed markedly in properties from the starting material. In a further effort to purify the material, the fractions *a*, *b*, *c* and *d* (Table 2) were mixed together and treated with 5% (v/v) trichloroacetic acid at 0°. The solution remained clear and, after neutralization, concentration *in vacuo*, dialysis and drying from the frozen state, gave a product which weighed 0·92 g. and contained 5·2% N. About a third of the material passed into solution on treatment with 90% phenol and was recovered by addition of ethanol to 10% (v/v) concentration. The precipitate was dissolved in a further quantity of phenol and again precipitated with ethanol at the same concentration. The material was finally dissolved in 90% phenol and fractionated from solution by ethanol within the concentration levels given in Table 3. The analytical figures and serological activities of the three middle fractions showed no significant variation (Table 3) and the materials were recombined. Electrophoretic examination of the material at pH 8·0 and 4·0 and ultracentrifugal analysis at

Table 2. *The fractionation of* Le^a *substance from phenol-glycol solution by ethanol*

Fraction	Precipitation level (% (v/v) ethanol)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)
a	0–8	0·05	−46°	4·3
b	8–11	0·28	−42°	5·2
c	11–14	0·33	−45°	4·9
d	14–20	0·27	−40°	5·1
e	20–excess	0·11	−38°	5·0

Table 3. *The fractionation from phenol by ethanol of* Le^a *substance after treatment with trichloroacetic acid*

Fraction	Precipitation level (% (v/v) ethanol)	Yield (g.)	Specific rotation $[\alpha]_{5461}$	N content (%)	Serological activity (% of standard preparation)
a	Phenol insoluble	0·58	−38°	5·3	100
b	0–5·0	0·08	−42°	5·0	150
c	5·0–7·5	0·11	−45°	5·0	150
d	7·5–10·0	0·13	−44°	5·0	150
e	10·0–excess	0·01	—	5·6	100

pH 8·0 failed to demonstrate inhomogeneity. The details of these analytical procedures are given by Dr R. A. Kekwick in an addendum to this paper.

A third ovarian cyst fluid, no. 157, obtained from a Lewis-positive, Le(a+), patient, measured about 8 l. and gave 320 g. of dry material, which, after extraction with 90% phenol by the usual technique, yielded a phenol-insoluble residue (29 g.; N, 7·1%) which was only partially soluble in water. The water-soluble component (14 g.; N, 6·1%) showed half the serological activity of the 'standard' Le^a preparation. This material was again extracted with 90% phenol and was now found to be appreciably soluble. The insolubility of the material in phenol at the first stage of the isolation procedure is presumably due to the presence of the fraction which is eliminated as a water-insoluble jelly. The phenol-soluble substance was fractionally precipitated from this solvent by gradual addition of ethanol. The material which separated from solution between the levels 0–10% ethanol was twice precipitated from phenol over the same range of ethanol concentration to give a product (1·4 g.; N, 5·8%; $[\alpha]_{5461} - 40 \pm 4°$) which was as active as the standard Le^a preparation. A solution of the substance in 0·2M-acetate, pH 4·6, was further deproteinized by repeated treatment with 1:1 (v/v) amyl alcohol/$CHCl_3$ as described earlier and gave a final product (1·1 g.; N, 5·6%; fucose, 11·5%; hexosamine, 33%; $[\alpha]_{5461} - 40 \pm 4°$) which showed only a single peak when examined in the electrophoresis apparatus at pH 8·0. This material has not yet been studied in greater detail.

Properties of the Le^a *substance*

Immunological. The two most active preparations of Le^a substance inhibited the serum 'Hughes' at a dilution of $1:10^6$. They were examined for their capacity to inhibit the following heterologous blood-group reagents: (1) Anti-H agglutinins (so-called anti-O) of animal and human origin (Schiff, 1927; Greenfield, 1928; Morgan & Waddell, 1945; Annison & Morgan, 1950). (2) Human anti-O serum (Boorman, Dodd & Gilbey, 1948; Morgan & Watkins, 1948). (3) Human anti-A and anti-B agglutinins. (4) Rabbit anti-M and N agglutinins. (5) Anti-P agglutinins. (6) Anti-*Rhesus* sera, representing anti-D, anti-C, anti-c and anti-E test reagents, and (7) anti-Le^b agglutinins. Tested at a concentration of 5 mg./ml., the best preparations showed no capacity to neutralize any of the test agglutinins.

The Le^a substance failed to induce in rabbits the formation of anti-Le^a agglutinins when given intravenously in several doses each of 0·1 mg. The substance showed no capacity to inhibit the haemolysis of sheep red cells by rabbit anti-A serum in the presence of complement and, in some tests made by Dr J. McCrea, failed to inhibit the haemagglutinating activity of heated LEE influenza virus. The material gave a precipitate with horse antipneumococcal type XIV serum and in giving this reaction differed in behaviour from the purified human A, B and H substances, which failed to precipitate with the type XIV reagent. The crude cyst fluid from a Lewis-positive patient gave rise to a precipitate with type XIV anti-serum.

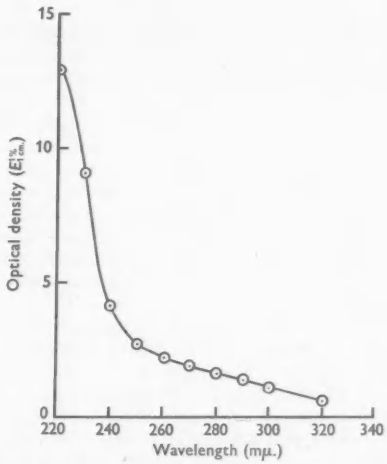

Fig. 2. The ultraviolet absorption spectrum of Le^a substance (0·1% solution in 0·85% NaCl).

Physical. The mean value of the specific rotation of the two most active preparations is $[\alpha]_{5461} - 41 \pm 4°$ (*c*, 1·0) in water. Relative viscosity determinations were made on the same two specimens dissolved in 0·85% sodium chloride, using a modified Ostwald viscosimeter at 37°; the values obtained, expressed in terms of the solvent, were: η, 1·36 and 1·38. These values are somewhat higher than those reported for A substance.

Solutions of the Le^a substances (0·10% in 0·85% NaCl) were examined in a Hilger quartz spectrophotometer (Uvispek) using a 2 cm. cell. The absorption curve for one specimen is given in Fig. 2. No significant amount of absorption is shown by the material between 260 and 300 mμ. and the absence of appreciable amounts of aromatic amino-acids and nucleic acid is indicated.

Chemical. A precipitate was not obtained when a 2% aqueous solution of the Le^a substance was treated with an equal volume of the following protein precipitants: trichloroacetic acid (10%, v/v), salicylosulphonic acid (20%, v/v), tannic acid, or picric acid. The Le^a substance likewise gave no precipitate on addition of solutions of lead acetate (in dilute acetic acid), mercuric acetate, aluminium sulphate in 5N-sulphuric acid, phosphotungstic acid, acid or neutral copper sulphate, or an acetic acid solution of potassium ferrocyanide.

The biuret, ninhydrin, xanthoproteic, Millon, Hopkins-Cole, Seliwanoff and Bial tests were negative. The Sakaguchi test for arginine, as modified by Keyser (1948), and the diazobenzenesulphonic acid test for tyrosine and histidine gave only a trace of colour when applied to a 2% solution of the Le^a substance.

The analytical figures obtained for the most active specimen of Le^a substance were: C, 41·5; H, 7·1; N, 5·3% (average Kjeldahl value). The phosphorus content was less than 0·02% and the sodium fusion test for sulphur indicated that this element was not present in appreciable amounts, a conclusion which is supported by the finding that cysteic acid and methionine were absent from the partition chromatograms of the total acid hydrolysate of the Le^a substance and that the unhydrolysed material failed to react with toluidine blue, a reagent used by Jorpes (1946) for the detection of polysulphate groups in polysaccharides.

The acetyl content of the Le^a substance was about 10% (6 hr. hydrolysis) and was in excess of that required for the *N*-acetylhexosamine calculated to be present from the total hexosamine value. The distillates obtained from the *N*-acetyl determinations were neutralized and were used to identify the volatile organic acid by means of an *S*-benzylthiouronium salt (Donleavy, 1936). The *S*-benzylthiouronium salt obtained in low yield after recrystallization from 80% ethanol melted at 134° and gave a mixed melting point of 135° with an authentic specimen of *S*-benzylthiouronium acetate.

The fucose content of the Le^a substance was about 12%. The maximum colour developed by the Le^a substance, after heating at 100° for 14 min. with dilute sodium carbonate (pH 10·8) and treatment with *p*-dimethylaminobenzaldehyde (Morgan & Elson, 1934), was equivalent to about 9% of *N*-acetylglucosamine. *N*-Acetylglucosamine required heating for 4 min. to develop its maximum colour under the same conditions.

The Le^a substance, after heating with dilute sodium carbonate for 14 min. at 100°, was dialysed against distilled water at 0°. The diffusate gave an immediate colour with the *p*-dimethylaminobenzaldehyde reagent, whereas the indiffusible residue gave no appreciable colour (cf. King & Morgan, 1944; Morgan, 1946). These results again demonstrate how closely in certain of its properties the Le^a substance resembles the human A substance.

Oxidation of the Le^a *substance with periodate*

The rate of reduction of periodate by the Le^a substance at pH 5·0 and 7·3 was measured by the techniques employed by Aminoff & Morgan (1951).

Oxidation at pH 5·0. A solution (0·1%) of the Le^a substance in 0·1 M-acetate, pH 5·0, was treated with 0·005 M-HIO_4. The rate of reduction of periodate and the loss of biological activity of the substance were determined. The results, shown graphically in Fig. 3, are closely similar to those obtained for the A substance under the same conditions. The analytical results, corrected for over-oxidation by extrapolating the uptake curve to zero time, showed that 1 mg. of Le^a substance reduces $0{\cdot}273 \times 10^{-5}$ g.mol. of HIO_4, and hence 365 g. of Le^a substance reduces 1 g.mol. of HIO_4.

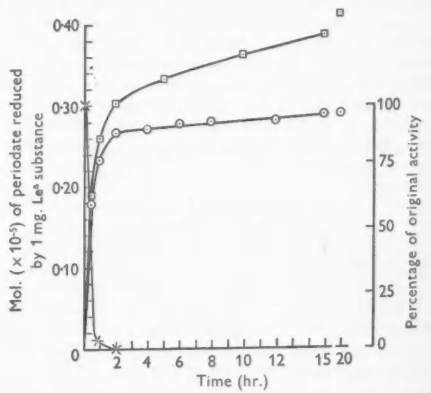

Fig. 3. Rate of reduction of periodate by Le^a substance at pH 5·0 (⊙) and pH 7·0 (⊡). The resulting inactivation at pH 5·0 as determined by the capacity of the oxidized material to inhibit the agglutination of Le (a+) cells by Le^a agglutinin (×).

Oxidation at pH 7·3. The rate of periodate uptake was greater at pH 7·3 than at pH 5·0 (Fig. 3). The uptake curve showed a less definite inflexion after 2 hr. and the periodate consumption continued steadily for several days. The serological activity of the substance was lost at approximately the same rate (Fig. 3) as at pH 5·0.

Acid hydrolysis of the Le^a *substance*

The Le^a substance was hydrolysed in sealed glass ampoules at 100° with (*a*) 0·5N-HCl, (*b*) 6N-HCl and (*c*) N-acetic acid. After heating under conditions *b* and *c* for the appropriate time the ampoules were cooled, opened and the contents evaporated to dryness *in vacuo* in small glass dishes over conc. H_2SO_4 and pellets of KOH. The products of hydrolysis with 0·5N-HCl (*a*) were neutralized with an equal volume of 0·5N-NaOH and not evaporated to dryness. The products of hydrolysis with 6N-HCl (*b*) were dark brown in colour and contained a precipitate of humin.

The course of hydrolysis with 6N-HCl was followed by measuring the α-amino N liberated. The results (Fig. 4) show that the liberation of α-amino N reached its maximum value in about 4 hr. and then represented 92% of the total N of the Le^a substance. The α-amino-acid N after 16 hr. hydrolysis was equivalent to about 37% of the total N. The α-amino N value includes the N liberated from the amino sugars set free during the hydrolysis.

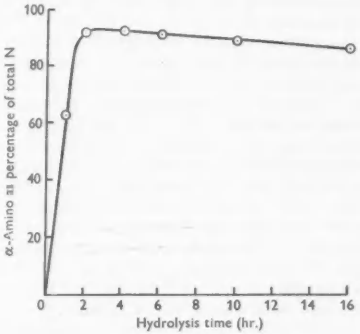

Fig. 4. Hydrolysis of Le^a substance by 6N-HCl at 100° as determined by the liberation of α-amino groups.

The liberation of reducing sugars and hexosamines is best followed by an examination of the products of 0·5N-HCl hydrolysis. The analytical results (Fig. 5) show that the maximum liberation of reducing sugars, 57% expressed as glucose, and of hexosamines, 32% expressed as glucosamine base, occurred at about the same time (6 hr.).

Hydrolysis of the Le^a substance with N-acetic acid at 100° allowed the rate and extent of liberation of fucose, reducing sugars and *N*-acetylhexosamine residues to be determined. The results (Fig. 6) indicate that a maximum value of about 13% fucose, determined as acetaldehyde after oxidation with periodate, is obtained after 24 hr. hydrolysis and a maximum value under these conditions for reducing sugars is reached at about the same time. There is an appreciable increase in the amount of '*N*-acetylglucosamine' colour given by the Le^a substance during the first 2 hr. of hydrolysis after which time the original value of 8·9%, expressed as *N*-acetylglucosamine, reaches a maximum value of 10·3% and then decreases.

During treatment with N-acetic acid at 100° the Le^a substance rapidly lost its capacity to inhibit iso-agglutination brought about by Le^a serum. At the same time there was a considerable enhancement of the pneumococcus type XIV character of the product. There was, however, no development in the products of a capacity to prevent the haemolysis of sheep cells by rabbit anti-A serum and complement.

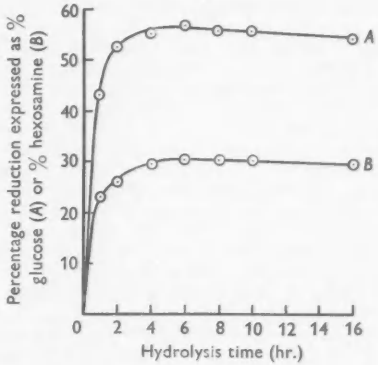

Fig. 5. Hydrolysis of Le^a substance by 0·5N-HCl at 100° as determined by (*A*) reducing power, expressed as glucose and (*B*) hexosamine liberated, expressed as glucosamine base.

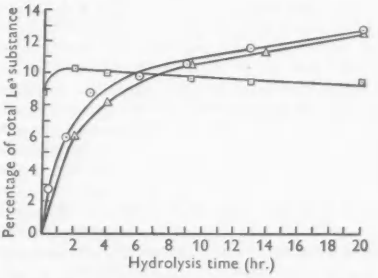

Fig. 6. Hydrolysis of the Le^a substance by N-acetic acid at 100° as determined by (⊙) reducing power, expressed as glucose, (△) '*N*-acetylhexosamine' colour, and (⊡) fucose, determined as acetaldehyde after oxidation with periodate.

Chromatographic analysis of the Le^a *substance*

A detailed investigation of the nature of the constituents of the Le^a substance was made by partition chromatography.

Amino-acids. The products of the complete hydrolysis (16 hr.) of the material (1 mg.) with 6N-HCl were put as a spot at one corner of a sheet of Whatman no. 1 paper. The

spot was exposed to NH_3 vapour and allowed to dry. The paper was then placed in the development tank together with a duplicate paper containing a similarly placed spot composed of an artificial mixture of those amino-acids believed as a result of a preliminary experiment to be present in the Le^a substance. *s*-Collidine saturated with water was used for the first run, which lasted 48–72 hr. The papers were then removed from the tank, dried at 37°, turned through a right-angle and irrigated for 24 hr. with a solution of water-saturated phenol (A.R.) in an atmosphere of coal gas containing NH_3. The positions of the individual amino-acids were revealed by spraying the papers with 0·1% solution of ninhydrin in butanol containing 1% (v/v) acetic acid (Consden & Gordon, 1948) and keeping the sheets at 37° for 1 hr. The papers were subsequently heated at 100° for 5 min. to detect the presence of peptides, but no additional coloured areas appeared. The chromatogram given by the complete hydrolysis products of Le^a substance gave rise to thirteen mauve spots on treatment with ninhydrin. Eleven of the spots on the chromatogram corresponded closely in position with those which were given by the following amino-acids: lysine, arginine, aspartic acid, glutamic acid, glycine, serine, alanine, threonine, proline, valine and leucine (isoleucine). The remaining greyish-mauve spots are not normally found in protein hydrolysis products and were subsequently identified as arising from glucosamine and chondrosamine.

Reducing sugars. The Le^a substance was hydrolysed with 0·5 N-HCl at 100° for 16 hr. and evaporated to dryness, and the chromatographic analyses of the sugar constituents were made by techniques essentially the same as those described by Partridge (1948). Strips of Whatman no. 1 paper (18 × 34 cm.) were employed as the inert supporting medium and the chromatograms were developed by irrigation with solvents run under gravity.

Five positions were marked across the width of each of eight filter paper strips and the following mixtures were put on each paper: (*a*) fucose, galactose and rhamnose; (*b*) the acid hydrolysate of Le^a substance; (*c*) the acid hydrolysate of Le^a substance together with fucose and galactose; (*d*) fucose, galactose and the two amino sugar hydrochlorides. Different sugars were introduced at position (*e*) as a further check on the resolving power of the solvent systems and in order to compare the colours produced by these sugars after treatment with aniline hydrogen phthalate with those produced by the sugar components of the acid hydrolysates of the Le^a substance. The eight paper strips were then irrigated in pairs with the following four solvent systems: (1) phenol-water, in an atmosphere of coal gas and HCN; (2) collidine-water; (3) *n*-butanol (40%), acetic acid (10%) and water (50%); (4) *n*-butanol-water, in an atmosphere of NH_3. The chromatograms were developed for 16–24 hr. with solvent systems (1) and (2), and for 36–48 hr. for the solvents (3) and (4). The period employed depended on the temperature of the laboratory during the irrigation. The strips were dried and one member of each pair was sprayed with $AgNO_3$-NH_3 reagent and the other with aniline hydrogen phthalate. No evidence was obtained for the presence in the Le^a substance of sugars other than fucose, galactose and the two hexosamines. A chromatogram of the hydrolysate of the Le^a substance was also sprayed with the naphthoresorcinol-trichloroacetic acid reagent (Partridge, 1948), when the absence of ketoses and uronic acids from the material was established.

Galactose (R_F, 0·44) can be readily distinguished from glucosamine (R_F, 0·62) and chondrosamine (R_F, 0·65) by virtue of its lower mobility in phenol, but fucose (R_F, 0·62) is not identifiable with this solvent, if a mixture of the four sugars is examined; this is so despite the fact that fucose reacts much more intensely than the hexosamines with aniline hydrogen phthalate. The use of *s*-collidine as the moving organic phase, however, allows fucose (R_F, 0·44) to be easily distinguished from the other three sugars. Galactose (R_F, 0·34) moves at almost the same rate as glucosamine (R_F, 0·32) in collidine, but can be readily detected (R_F, 0·16) between fucose (R_F, 0·27) and the amino sugars (R_F, 0·12–0·13) on chromatograms irrigated with butanol-acetic acid-water.

The $AgNO_3$-NH_3 reagent for the detection of sugars is somewhat unsatisfactory, since it is applied in the form of an aqueous solution and some migration of the sugar-containing areas may occur if the chromatogram is not lightly and evenly sprayed. Furthermore, Cl^- migrates at speeds comparable to those of the sugars in some solvents and under these circumstances reacts with the reagent to give a heavy white precipitate of AgCl which masks the brown-coloured areas given by the reducing sugars. Fortunately, the aniline hydrogen phthalate reagent is applied to the chromatogram in butanol and does not give rise to noticeable migration of the sugar-containing areas, and furthermore the presence of Cl^- does not interfere with the brown coloration given by reducing sugars.

The amino sugars. The investigations of Aminoff & Morgan (1948) showed that a good separation of the amino sugars on paper is facilitated by the use of alkaline solvents, such as *s*-collidine and *n*-butanol-NH_3. Chromatograms were therefore run with the hydrolysis products of Le^a substance, alone and admixed with glucosamine and chondrosamine, using these solvents for irrigation. Five positions were marked out along the width of each of two filter paper strips (18 × 34 cm.) and the following substances were put on each paper in the order given: (*a*) glucosamine hydrochloride, (*b*) glucosamine and chondrosamine hydrochlorides, (*c*) acid hydrolysis products of Le^a substance, (*d*) acid hydrolysis products of Le^a substance with (*b*) added, and (*e*) chondrosamine hydrochloride alone. The chromatograms were developed in parallel with *s*-collidine as solvent for 48 hr. and then dried. One strip of each pair was sprayed with the ninhydrin reagent and the other successively with the two hexosamine reagents. The chromatograms indicated that glucosamine and chondrosamine are components of the Le^a substance.

The two-dimensional chromatograms of the hydrolysis products of the A substance and the Le^a substance revealed that, whereas the glucosamine- and chondrosamine-containing areas derived from the A substance were almost identical in size and colour intensity, the glucosamine spot given by the hydrolysis products of the Le^a substance was two to three times as large the chondrosamine area.

The possibility that the second amino sugar spot was due to *N*-methyl-L-glucosamine was rendered improbable when it was shown that the latter substance possesses a slightly greater mobility in *s*-collidine than glucosamine and gives colours with the ninhydrin and hexosamine reagents which differ from those produced by the two unsubstituted amino sugars, glucosamine and chondrosamine.

The isolation of the component sugars

The sugars were isolated by partition chromatography on cellulose columns according to the general procedure described by Hough, Jones & Wadman (1949). The results of preliminary experiments with paper chromatograms showed that fucose and galactose could be separated from each other and from the amino sugars also present in the acid hydrolysates of the Le^a substance, if the solvent system 1:1 *n*-butanol/2% aqueous acetic acid was employed.

All melting points given below are uncorrected.

Le^a substance (500 mg.) was hydrolysed in sealed tubes with 0·5 N-H_2SO_4 at 100° for 8 hr. The hydrolysate was neutralized to pH 7 with saturated $Ba(OH)_2$ and the precipitated $BaSO_4$ removed by centrifugation and thoroughly washed with water. The solution and washings were combined and taken to dryness *in vacuo*. The residue was dissolved in a few drops of water and placed on the top of a cellulose column (35 × 2·5 cm.) which had been previously washed by the passage of 500 ml. of the organic phase of the *n*-butanol/2% aqueous acetic acid system. The chromatogram was allowed to develop under gravity and 4 ml. portions of the solvent issuing from the column were collected. The sugars were detected and identified as described by Hough *et al.* (1949). The fucose was readily obtained completely free from galactose and the amino sugars and most, but not all, of the galactose emerged from the column uncontaminated with the amino sugars.

L-*Fucose* as *diphenylhydrazone*. The fractions issuing from the column which contained fucose were evaporated to dryness *in vacuo* at 30–40°. The residue was dissolved in water (2 ml.), and glacial acetic acid (0·2 ml.) and freshly distilled diphenylhydrazine (60 mg.) were added. The solution was allowed to stand overnight in the dark and at room temperature, and was then cooled, when 35 mg. of needle-shaped crystals separated. The derivative was twice recrystallized from 95% (v/v) ethanol when 19·5 mg. of material melting at 201° was obtained. Mixed melting point with authentic fucose diphenylhydrazone remained unchanged. (Found: C, 65·1; H, 6·5; N, 8·7. Calc. for $C_{18}H_{22}O_4N_2$: C, 64·7; H, 6·7; N, 8·5%. $[\alpha] - 13°$ in pyridine (*c*, 1).)

D-*Galactose* α-*methylphenylhydrazone*. The fractions collected which contained galactose were mixed and evaporated to dryness *in vacuo*. The crystalline residue was dissolved in water (2 ml.), and freshly distilled α-methylphenylhydrazine (0·1 ml.), ethanol (2 ml.) and 10% acetic acid (0·1 ml.) were added. The mixture was kept at 37° for 2 hr. and overnight at 0°. D-Galactose α-methylphenylhydrazone (59 mg.) separated and after recrystallization from 30% (v/v) ethanol gave 48 mg. of needle-shaped crystals m.p. 191°. Mixed melting point with authentic D-galactose α-methylphenylhydrazone 191°. Found: C, 53·8; H, 7·4; N, 10·2. Calc. for $C_{13}H_{20}O_5N_2$: C, 54·9; H, 7·0; N, 9·9%.)

D-*Galactose* α-*methylphenylhydrazone pentaacetate*. Galactose α-methylphenylhydrazone (20 mg.) was suspended in 1 ml. of dry pyridine and 0·2 ml. of acetic anhydride added at 0° (Wolfrom & Christman, 1931). The mixture was allowed to stand overnight at 0°, and, with frequent shaking, at room temperature for a further 24 hr. The solution was then poured into excess chilled water and the white crystalline material was collected and well washed with water. The material after recrystallization from 50% ethanol gave 29 mg. of D-galactose α-methylphenylhydrazone pentaacetate. $[\alpha]_D + 36 \pm 2°$ in 95% ethanol (*c*, 1); m.p. 139–140° without decomposition. Mixed melting point with an authentic specimen of the pentaacetate remained unchanged.

N-2:4-*Dinitrophenylamino sugars*. A method for the separation and identification of small amounts of a mixture of glucosamine and chondrosamine as dinitrophenyl (DNP) derivatives has been described by Annison, James & Morgan (1950, 1951). The yields of the DNP derivatives obtained from known amounts (10–20 mg.) of the amino sugar hydrochlorides and recovered from the kieselguhr column were of the order of 80%. The results of qualitative paper chromatography had shown that the Le^a substance contained the amino sugars, glucosamine and chondrosamine, in the ratio of about 3:1. In order, therefore, to isolate sufficient DNP-chondrosamine to allow the material to be purified by crystallization and analysed, it was necessary to hydrolyse 500 mg. of the Le^a substance. The yield of the mixed DNP-amino sugars represented 69% of the total hexosamine present in the Le^a substance. The DNP-glucosamine weighed 97·4 mg. and the DNP-chondrosamine 36·3 mg. The ratio of glucosamine to chondrosamine was 1:2·7. The isolated DNP-glucosamine had melting point 197–200° and mixed melting point with authentic DNP-glucosamine 199–201°. $[\alpha]_{5461} + 68 \pm 4°$ in 80% ethanol (*c*, 1). (Found: C, 42·0; H, 4·6; N, 12·0. Calc. for $C_{12}H_{15}O_9N_3$: C, 41·7; H, 4·4; N, 12·2%.) Isolated DNP-chondrosamine had melting point 177–180° and mixed melting point with authentic DNP-chondrosamine 178–180°. $[\alpha]_{5461} + 82 \pm 4°$ in 80% ethanol (*c*, 1). (Found: C, 41·5; H, 4·4; N, 11·9. Calc. for $C_{12}H_{15}O_9N_3$: C, 41·7; H, 4·4; N, 12·2%.)

The examination of the products of partial hydrolysis of Le^a *substance*

The products of the hydrolysis of 50 mg. of Le^a substance with N-acetic acid at 100° for 48 hr. were dialysed in a thoroughly washed cellophan bag for 7 days at 0° against frequent changes of distilled water. The diffusates were stored at 0° in the presence of a few drops of $CHCl_3$ until the dialysis was complete. The total diffusates were then mixed together, concentrated to a small volume and dried from the frozen state. The indiffusible contents of the cellophan bag were likewise dried. The diffusible material (16 mg.) was obtained as a hygroscopic glass, the indiffusible material (17·1 mg.) as a light-brown friable solid. Fucose, galactose, an *N*-acetylhexosamine and traces of glucosamine and chondrosamine were detected by chromatography in the diffusate. The solvents employed, however, were not chosen to resolve a mixture of the *N*-acetylhexosamines. The developed chromatogram of the diffusate when sprayed with aniline hydrogen phthalate indicated the positions of fucose and galactose only. $AgNO_3$-NH_3 gave in addition three other brown spots associated with slowly moving substances which were presumably due to the presence in the diffusate of partial hydrolysis products of the polysaccharide moiety of the Le^a substance. Two of these unidentified spots reacted with the hexosamine reagents.

The presence in the diffusate of a small amount of an amino-acid or peptide which moved slowly in all the solvents used was revealed when the chromatograms were sprayed with ninhydrin. This spot and the other three unidentified

spots which appeared on treatment with $AgNO_3$-NH_3 reagent were no longer present after the diffusate was hydrolysed further with 0·5 N-HCl for 16 hr. at 100°. Fucose, galactose and the two amino sugars were then detected. The ratio of glucosamine to chondrosamine in the diffusate, as judged by the areas of the spots produced, was about 5:1. The chromatographic analyses of the diffusate after hydrolysis with 6 N-HCl showed that traces of all the amino-acids except proline, leucine and arginine were present.

The non-diffusible material was hydrolysed with 0·5 N-HCl at 100° for 16 hr. and examined chromatographically for sugars. Fucose, which is stable under the conditions of hydrolysis employed, was not detected in these analyses, but galactose, glucosamine and chondrosamine were identified. The ratio of glucosamine to chondrosamine was estimated from the areas of the spots produced to be about 3:2.

The amino-acid distribution in the indiffusible material was investigated after further hydrolysis with 6 N-HCl for 16 hr. at 100° and compared with that given by the original, fully hydrolysed material. No significant difference could be detected.

DISCUSSION

The results of the investigations described show that the Lewis Le^a substance is a mucoid material which possesses physical and chemical properties very similar to those recorded for the other water-soluble products of the human blood-group genes. Whereas one might expect that the products of allelomorphic genes would closely resemble each other chemically, it is perhaps surprising that the products of genes genetically independent should be so similar. This emphasizes the importance of determining the precise differences in the nature of these gene products for our understanding of the mechanism of gene action.

Ovarian cyst fluids have been used as a source of Le^a substance and by selecting cyst fluids obtained from Lewis-positive, Le(a+), women it is possible to avoid the presence of A, B, H and Le^b substances in the starting material. Once again it has been found that the variable amount of the Le^a substance present and the unknown nature of the accompanying unspecific materials in the native cyst fluids leads to an unpredictable behaviour of the crude material and an exact description of an isolation procedure which leads to an homogeneous product and can be applied successfully to all cyst fluids cannot be given.

The blood-group substances in dried, native ovarian cyst fluids are usually found to be insoluble in 90% phenol. A preliminary treatment of the material with this solvent, however, readily removes a considerable part of the unspecific protein and leaves a mucoid material which possesses almost the whole of the blood-group activity of the native fluid. It has been found that the phenol-insoluble mucoid still contains a small amount of material which from its nitrogen content appears to be largely protein and this impurity can be most easily and completely removed, if the substance is fractionally precipitated from solution in 90% phenol. The problem of getting the serologically active mucoid into solution in 90% phenol has been only partially solved. It has been observed, however, that after the removal of accompanying unspecific material the specific mucoid frequently becomes appreciably soluble in 90% phenol and can be recovered from solution in this solvent by addition of ethanol. Two other procedures have also been found which will bring about the solution of the specific mucoid in 90% phenol. The first consists in treating the native material with pepsin at pH 2·3 and 37° for several days. This method has been used extensively by Kabat and his co-workers (see Kabat, 1949), but for reasons which will be given elsewhere we have not used the method, believing that prolonged peptic hydrolysis brings about a change in the blood-group substance. The second method involves treatment of an aqueous solution of the phenol-insoluble material with 5% trichloroacetic acid at 0° for a short time. A part of the substance is subsequently found to be soluble in 90% phenol and can be recovered from this solvent by addition of ethanol to a low final concentration. Irrespective of group specificity the phenol-insoluble mucoid materials so far investigated are found to dissolve, in part and in the cold, in 90% phenol which contains 20% ethyleneglycol. The soluble component, which exhibits a much increased serological activity, can be recovered from this and other similar organic solvent systems by addition of excess phenol or ethanol.

The application of the different techniques for the isolation of the blood-group mucoid gives a material of high Le^a activity which appears to be essentially homogeneous on the basis of its behaviour during electrophoresis. The electrophoretic diagrams obtained for periods of observation up to nearly 5 hr. at pH 4·0 and 8·0 fail to reveal the presence of more than a single component. No appreciable migration of the Le^a substance takes place at pH 4·0, but a slow movement towards the anode is detected in buffer solution at pH 8·0. Examination in the ultracentrifuge at pH 8·0 showed that only one main component was present, but the material was nevertheless moderately polydisperse. The mean sedimentation constant at a concentration of 0·5% is S_{20}, $6{\cdot}7 \times 10^{13}$. From the ultracentrifugal and diffusion data the particle weight of the Le^a substance is of the order 270,000.

The application of the usual techniques of paper chromatography to the products of acid hydrolysis of the Le^a substance demonstrates that fucose, galactose, glucosamine and chondrosamine are component sugars and that at least eleven amino-acids are also present in the native complex. The

chromatograms are indistinguishable qualitatively from those given by the group A substance. The aromatic acids, phenylalanine and tyrosine, are absent, whereas threonine occurs in a higher concentration than any of the other amino-acids. Sulphur and phosphorus are not present in appreciable amounts, and the Le^a substance shows no characteristic absorption bands in the ultraviolet region (210–300 mμ.).

The results of the quantitative determination of the component sugars in the different blood-group substances indicates that the amounts of the sugar residues are different in the different gene products. A substance, for example, contains about 18% fucose, three-quarters of which is liberated as free sugar after hydrolysis with acetic acid (pH 2·8) at 100°, whereas Le^a substance contains only some 13% fucose, almost the whole of which is split off under the same conditions of hydrolysis. The ratio of D-glucosamine to D-chondrosamine in the group A substance is very close to one, whereas these two amino sugars are found together in the Le^a substance in the ratio of about 3:1. The low yield of *S*-benzylthiouronium acetate obtained from the distillate collected during the quantitative determination of acetic acid liberated by the Le^a substance does not allow the presence of other volatile organic acids to be excluded.

Kabat, Bendich, Bezer & Knaub (1948) reported that the A, B and O (H) substances obtained from hog and human sources precipitate with antipneumococcal type XIV horse serum. Our preparations of these specific substances obtained from human ovarian cysts fail to react with this antibody (Aminoff *et al.* 1950 and unpublished observations). In view of the finding now reported that Le^a substance reacts with antipneumococcal type XIV serum it seems probable that human erythrocytes belonging to all groups within the ABO classification are agglutinated by this antiserum, as first reported by Finland & Curnen (1938), Weil & Sherman (1939) and Goebel, Beeson & Hoagland (1939), not by virtue of their A, B and H receptors but rather through the reactivity of other components of the red cell surface, such as through the presence of the product of the *Le*^{a} gene. Specific substances responsible for other serological group characters, and especially that derived from the closely related 'Lewis' *Le*^{b} gene, might well react with pneumococcal type XIV antibody. In most instances, however, the chemical nature of the substances associated with these other group characters, such as M, N, P, *Rhesus*, etc., is unknown. Some indirect evidence from a study of the action of periodate on the erythrocyte surface (Morgan & Watkins, 1951) suggests that these receptors might be due to carbohydrate-containing materials, and therefore could possibly contain the basic glucosamine-galactose structure which, according to Kabat, Baer, Bezer & Knaub (1948), is responsible for the serological cross-reactivity observed. The hypothesis, however, cannot be substantiated until a more detailed examination of the reactivity of a much larger number of undegraded blood-group substances with the type XIV antiserum has been undertaken.

The examination of the genetical schemes (see Grubb & Morgan, 1949; Race & Sanger, 1950; Andresen, Andersen, Jordal & Henningsen, 1950; Grubb, 1951) elaborated to explain the serological behaviour of erythrocytes, of tissue fluids and secretions indicates the complex nature of the Lewis group system and it must be admitted that the genetics of this system are not completely understood. According to Grubb (1951) it seems probable that the serological observations can be accounted for, if four genes, existing as two allelomorphic pairs, *Le*^{a}–*Le*$^\alpha$ and *Le*^{b}–*Le*$^\beta$, are each responsible for inducing the formation of a gene product which contributes to the ultimate serological 'Lewis' character of the individual's erythrocytes and secretions. It is not known if the gene products Le$^\alpha$ and Le$^\beta$ actually exist, but if they do Le$^\alpha$ substance will be encountered in rather less than half the fluids obtained from 'Lewis' positive persons, whereas all such fluids will contain Le$^\beta$ substance. Since it is generally believed that the products of allelomorphic genes are basically similar, it would seem probable that the product of the gene *Le*$^\alpha$ is closely related chemically to that of the gene *Le*^{a} which is now known to be a mucoid. In the absence of serological reagents for the detection of Le$^\alpha$ and Le$^\beta$ substances these materials would remain unidentified serologically if they are present in preparations of Le^a substance.

The establishment of homogeneity of material of large particle size is a matter of considerable difficulty, and in spite of the apparently satisfactory nature of the results of a number of homogeneity tests which have been applied to the Le^a preparations involving the use of physical, chemical and immunological measurements, it is evident that the similarity of the products of allelomorphic genes and the absence in some instances of specific serological reagents for their detection and complete characterization suggest caution in concluding that the Le^a preparations described are completely homogeneous. It is to be observed that the quantitative immunochemical technique developed by Kabat *et al.* (see Kabat, 1949), although a valuable procedure for the measurement of homogeneity in many instances, is not necessarily immediately applicable to every problem of the kind. Sera suitable for quantitative precipitation tests in the blood-group field have to be made largely by the immunization of man or animals. The immunizing antigens, whether they are red cells or native secre-

tions, such as saliva or partially purified group specific substances, almost certainly contain antigens in addition to the one under investigation. The immune serum formed would most probably possess antibodies specific for the antigens injected and precipitate with all or some of the components of the original 'antigen' preparation. A serum with these properties is not a suitable reagent for use in the quantitative immunochemical technique.

The particle sizes of the human blood-group substances which have been isolated so far are such that one might expect that a strictly limited variation in composition or structure would be encountered in group materials obtained from individuals whose erythrocytes and secretions ostensibly show the same serological specificity. Indeed, it seems probable that only a part of a group substance whose particle size is as large as 250,000 need possess the specific configuration necessary to bring about its combination with homologous antibody. The remaining part of the group substance could presumably vary to some extent as long as the change brought about no demonstrable influence on the closeness of fit of the substance with the antibody concerned. At this early stage in the development of our knowledge of the human blood-group substances, however, every effort should be made to obtain these materials uncontaminated with other substances of closely similar composition and origin, as far as it is possible to do so by employing in a critical manner the physical, chemical and immunological techniques at present available, before it is concluded that similar group characters in different individuals are due to materials possessing a different chemical composition.

SUMMARY

1. The isolation and purification of the human blood-group Le^a substance is described.

2. The substance is a laevorotatory mucoid. The mucoid is slightly polydisperse, but appears to be essentially homogeneous on the basis of a number of physical, chemical and immunological properties.

3. L-Fucose, D-galactose, D-glucosamine, D-chondrosamine and eleven amino-acids have been identified as components of the Le^a substance.

4. The products of acid hydrolysis of the Le^a substance contain 57% of reducing substances, expressed as glucose, 13% of L-fucose and 32% of hexosamine. The α-amino-acid nitrogen represents about 37%, and the α-amino nitrogen 92%, of the total nitrogen (5·0%).

5. Evidence for the homogeneity of the Le^a substance is discussed and the possible presence of other mucoids is recognized.

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ADDENDUM

Physicochemical Examination of Lewis (Le^a) Blood-group Substance

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The Lewis (Le^a) substance was examined in the electrophoresis apparatus and ultracentrifuge, and in addition the partial specific volume and diffusion constant were determined.

METHODS

Electrophoresis. The material was examined in a Tiselius electrophoresis apparatus at 0°, using the diagonal schlieren optical system and monochromatic light, $\lambda = 546$ mμ., isolated from a mercury arc by a suitable filter. As the main object of this examination was to detect any traces of contaminating high molecular substances, solutions of salt-free material were made up in suitable buffers to give a concentration of 2 g./100 ml. and dialysed against buffer overnight.

Sedimentation. The ultracentrifugal examination was carried out in a Svedberg oil turbine machine at 60,000 rev./min. (270,000 *g*) using an optical system similar to that of the electrophoresis apparatus. The variation of sedimentation constant with concentration was examined over a limited concentration range. Solutions were made up in suitable buffers and sufficient NaCl was added to suppress any charge effect on sedimentation.

Diffusion. The diffusion measurements were made by Dr A. G. Ogston, using the method of Coulson, Cox, Ogston & Philpot (1948).

Partial specific volume. This was determined pyknometrically at 25°, the substance being dissolved in the buffer used for the majority of the sedimentation constant measurements.

RESULTS

In acetate, pH 4, $I = 0{\cdot}2$ and in phosphate pH 8, $I = 0{\cdot}2$, after prolonged electrophoresis only one component was demonstrable. At pH 4 the boundaries were symmetrical, but at pH 8 both ascending and descending boundaries were asymmetrical, and the peaks were partially obscured by a small amount of material associated with marked light scattering (Fig. 1). Migration at both pH 8 and 4 was anodic, but very slight at pH 4.

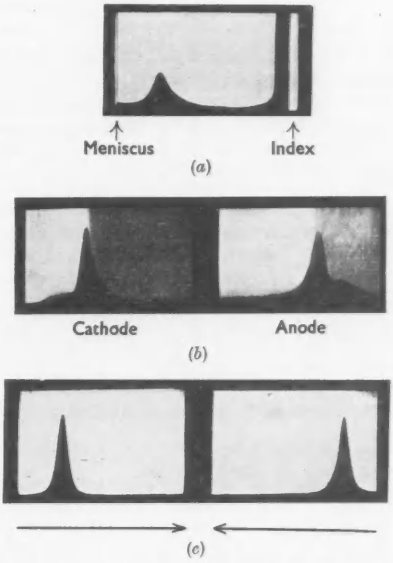

Fig. 1. Ultracentrifuge and electrophoresis diagrams of Lewis (Le^a) substance. (*a*) Ultracentrifuge diagram of 1% (w/v) solution in phosphate-NaCl (phosphate pH 8, $I = 0{\cdot}2$, 0·15 M-NaCl) after 50 min. at 270,000 *g*. (*b*) Electrophoresis diagram of 1·6% (w/v) solution in phosphate (pH 8, $I = 0{\cdot}2$) after 270 min. at 5 V./cm. (*c*) Electrophoresis diagram of 2% (w/v) solution in acetate (pH 4·0, $I = 0{\cdot}2$) after 300 min. at 5 V./cm. Migration direction in (*b*) and (*c*) shown by arrows.

In the ultracentrifuge only one component was present. The sedimentation constant was determined at three concentrations (Table 1). Owing to the spreading of the boundary it was not possible to make satisfactory measurements below a concentration of 0·5 g./100 ml. There is a marked dependence of sedimentation constant on concentration.

In Table 1 is included a value for the diffusion constant, kindly measured by Dr A. G. Ogston, at a concentration of 1 g./100 ml. The partial specific volume was found to be 0·643.

Table 1. *Sedimentation and diffusion constants of Lewis blood-group substance*

(Sedimentation and diffusion constants corrected to water at 20°.)

Concentration (g./100 ml.)	$S_{20} \times 10^{13}$ (corr.)	$D_{20} \times 10^{7}$ (corr.)
0·50	6·71	—
0·75	6·27	—
1·00	5·44	1·37

From the sedimentation and diffusion constants determined at a concentration of 1 g./100 ml., and the partial specific volume, a molecular weight of 270,000 was obtained from the equation

$$M = \frac{RTS}{D(1 - V\rho)},$$

where M = molecular weight, R = gas constant, S = sedimentation constant, D = diffusion constant, T = absolute temperature, V = partial specific volume, and ρ = density of solvent (Svedberg & Pederson, 1940).

From the same data the frictional ratio f/f_o was computed, a value of 3·8 being obtained. This corresponds to an axial ratio of about 90 (Svedberg & Pederson, 1940); alternatively this value may be interpreted as indicating a high degree of hydration.

DISCUSSION

The characteristics of the Lewis blood-group substance are very similar to those previously reported for the blood-group A substance (Kekwick, 1950). Electrophoretically the substance is essentially homogeneous, which indicates that the protein nitrogen of the material is not due to contamination with extraneous protein, but is an integral part of the molecule. The ultracentrifuge data suggest a moderate degree of polydispersity, since the spread of the sedimenting boundary is greater than would be expected for a homogeneous substance of molecular weight 270,000 also showing the rather marked dependence of sedimentation constant on concentration found in this instance. The data are insufficient to give a precise expression of the range and distribution of molecular size. The molecule is probably either highly asymmetrical or highly hydrated.

SUMMARY

1. The Lewis Le^a blood-group substance is essentially homogeneous electrophoretically.

2. The mean molecular weight of the substance calculated from diffusion and sedimentation data is 270,000, and the frictional ratio 3·8.

3. The substance is probably moderately polydisperse and the molecular shape highly asymmetrical, or alternatively the molecule is highly hydrated.

I wish to acknowledge the technical assistance of Mr H. Murray.

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The Effect of Compounds Allied to Resorcinol upon the Uptake of Radioactive Iodine (^{131}I) by the Thyroid of the Rat

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Bull & Fraser (1950) observed the induction of myxoedema and goitre by the prolonged application of resorcinol ointment to varicose ulcers, subsidence of the myxoedema on withdrawal of the ointment, and lowering of the thyroid function on its re-application. The antithyroid action of resorcinol in rats was established by Doniach & Fraser (1950), who found that subcutaneous injection of the drug considerably lowered the thyroid iodine uptake in a manner comparable with that of methylthiouracil. The experiments reported below were undertaken in sequel to this work, in an attempt to determine whether this antithyroid activity is specific to recorcinol, or whether it is exhibited by related compounds.

EXPERIMENTAL

Materials. A number of mono- and poly-hydric phenols and their derivatives were selected from compounds commercially available, purity being checked by melting point determinations. These agreed to within 5° with the figures quoted in the literature, except in the cases of 2:7-dihydroxynaphthalene (185°), gallic acid (240°) and *p*-hydroxybenzoic acid (220°), where the melting points experimentally found are given in parentheses.

Hydroxyquinol and its triacetate were synthesized according to the method of Thiele (1898). The former melted correctly at 140°, the latter at 91·5°, 5° below the figure quoted by Vliet (1941).

Methods. The method used for assay of antithyroid activity in the rat was similar to that described by Doniach & Fraser (1950). The compounds were tested in small groups, each group with its own controls, on a total of thirty-five experimental days spread over a period of 1 year. Since the uptake of iodine by the thyroid of the rat varies with environmental temperature, diet and size of the animal, we concentrated on standardizing these conditions for each experimental day rather than for the whole period, and only made direct comparisons of iodine uptake within any one group of controls and treated rats. In most experiments, female albinos of average weight 150 g. were used, four for each compound and four controls.

The compounds were administered by subcutaneous injections of 1 ml. of solution in water (or, where necessitated by reason of insolubility, in 1:5 ethanol-water mixture), mostly at two dose levels, 0·25 and 0·1 m-mole/rat. This was equivalent to 180 and 70 mg./kg./rat in the case of resorcinol. In one experiment 0·06 m-mole of compound per rat and in another 0·125 m-mole were used. In some cases 2 ml. of solvent were needed, and in a few cases suspensions instead of solutions had to be used.

Controls received a subcutaneous injection of ethanol-water mixture in those few groups where it was used as a solvent for the drugs. To provide an additional standard for comparison a group of four rats injected with resorcinol was included on most experimental days.

Ten to twenty minutes after receiving the compound each rat was given an intraperitoneal injection of 1 ml. of distilled water containing 10 μc. of carrier-free radioactive ^{131}I, of half-life 8 days, in the form of NaI. The controls were similarly injected and all rats were killed about 2 hr. later. The short time of exposure to the compounds of 2·5 hr. was chosen purposely, because of the known rapid metabolism and excretion of the phenols. Owing to difficulties in exact timing, the treated rats were killed at an average 2 hr. 5 min., and the controls (usually dealt with last) 2 hr. 10 min., after receiving ^{131}I.

The thyroids were removed attached to the trachea, and each was digested overnight at 60° in a 10 ml. screw-capped bottle containing 5 ml. ethanolic NaOH (5 g. NaOH (A.R.), 50 ml. distilled water, ethanol to 100 ml.). After cooling, 5 ml. water was added to each bottle, the mixed contents of which were poured into a liquid counter, type M. 6 (CV 2145) (Twentieth Century Electronics) and the radioactivity was determined. The rate of count varied from about 500 to 5000/min. All the samples from any one experimental group were counted in one session. For the calculation of means and standard errors the results were finally recorded to the nearest 10 counts/min. Comparisons were then made between the figures of the treated and the control rats in each group.

RESULTS

The experimental findings, of which one detailed example is given in Table 1, are summarized in Tables 3–6. It is seen from Table 1 that the variation in iodine uptake in similarly treated rats is sufficiently marked to mask any possible correspondence with animal weight. The average time of exposure of the treated animals to the radioactive iodine in this case was 2 hr. 10 min. and for the controls 2 hr. 21 min. That this small difference in time did not significantly influence ^{131}I uptake is shown by the fact that the count rate in one rat treated with the inactive (i.e. non-antithyroid) compound, 2:3-dihydroxynaphthalene, exceeded three of the control figures. Similar individual overlaps occurred in all experiments which included inactive drugs. A factor which may well have lowered the mean count rate in the treated animals is the likely non-specific toxic reaction of phenols in all tissues including the thyroid.

Table 1. *The effect of various compounds on* ^{131}I *uptake by the thyroid gland. Sample experiment*

(Dose, 0·1 m-mole/rat, all female albinos.)

Treatment	Rat weight (g.)	Time (min.): Between injection of compound and of ^{131}I	Time (min.): Between injection of ^{131}I and killing	Counts $\times 10^{-1}$/min.: Individual values	Counts $\times 10^{-1}$/min.: Mean ± S.E.	Thyroid uptake of ^{131}I (as % of controls)	P*	Specific antithyroid activity
Thymol	170	14	121	202	272±44	63	0·05–0·02	Nil
	175			337				
	180			357				
	160			190				
2:3-Dihydroxynaphthalene	170	19	127	186	308±61	72	>0·05	Nil
	170			319				
	170			255				
	160			470				
Resorcinol	160	18	131	62	47±7·8	11	<0·001	Marked
	160			33				
	170			59				
	160			34				
p-Cresol	165	17	136	240	252±25	59	0·01–0·001	Slight
	155			191				
	155			264				
	175			311				
2:4-Dihydroxybenzoic acid	150	15	135	83	103±16	24	<0·001	Marked
	160			144				
	165			112				
	170			73				
Pyrogallol	160	14	140	198	232±30	54	0·01–0·001	Slight
	170			196				
	165			212				
	165			322				
Controls	175	0	141	470	429±31	100	—	—
	170			339				
	170			465				
	175			444				

* P = the probability that the difference between the means of the control and treated groups occurred by chance. This figure was derived from Student's t test.

The significance of difference between the count rates of each group and those of the controls was calculated by Student's t test, and the probability that such a difference was due to chance was recorded in terms of probability P derived from Fisher & Yates (1948). For convenience of tabulation we have converted the ratios between the mean ^{131}I uptakes of treated and control rats into approximate percentages.

It is seen in Table 1 that resorcinol produced a reduction in iodine uptake to about 11% of the control figure, followed in order of activity by 2:4-dihydroxybenzoic acid which reduced uptake to about 24%. The figures for P are influenced by degree of scatter, as well as by lowering of thyroid uptake, and this scatter tends to push results to the inactive side. Since, however, we must take into consideration the non-specific toxicity which may lower the thyroid uptake of all treated animals, we have only accepted as antithyroid those drugs which cause a reduction in iodine uptake to 50% or less with a $P<0·05$. In most cases where the means show a twofold or greater reduction, P was 0·01 or less. We have not regarded as antithyroid those drugs which show a significant ($P=0·05–0·02$), but only slight, reduction in iodine uptake, e.g. thymol in Table 1. We have also considered the results of repeated experiments and the effect of differing concentrations of the compounds (Tables 3–5), in the final assessment of antithyroid activity (Table 6).

Toxic effects. Most of the compounds, both active and inactive, proved toxic when given at a dose of 0·25 m-mole/rat. The toxic effects, which were either absent or minimal in the 0·1 m-mole dose, were manifested as an intense shivering lasting in the worst cases (e.g. catechol) up to 1 hr. followed by 15–30 min. lethargy, or as a lethargy lasting up to 1 hr. from the outset. All the dihydroxynaphthalenes used in these experiments, at a dose of 0·25 m-mole/rat, produced a deep narcosis lasting up to 2 hr., the animals remaining warm during the whole period. In the strength of 0·1 m-mole/rat

Table 2. *Effect of narcosis and shock on thyroid iodine uptake*

Treatment	Experiment no. 33: Thyroid iodine uptake (as % of controls)	33: *P*	34: Thyroid iodine uptake (as % of controls)	34: *P*	35: Thyroid iodine uptake (as % of controls)	35: *P*
Resorcinol (0·1 m-mole/rat)	—	—	14	<0·001	—	—
Controls	100	—	100	—	100	—
$MgSO_4.7H_2O$ (0·75 mg./100 g.)	92	>0·05	—	—	—	—
Nembutal anaesthesia	—	—	81	>0·05	—	—
$MgSO_4$ + ATP	58	0·01–0·001	—	—	40·5	<0·001
Ether anaesthesia	—	—	60	0·05–0·02	—	—

Table 3. *Effect on thyroid iodine uptake of monohydric phenols and certain derivatives*

(All experiments were on groups of four rats unless otherwise stated.)

Name of compound	m-moles/rat	Thyroid uptake of ^{131}I (%)	*P*
Phenol	0·25	34	0·01–0·001
	0·25	40	>0·05
	0·25	45	0·05–0·02 (8 rats)
	0·25	53	0·01–0·001 (8 rats)
	0·1	>100	>0·05
o-Cresol	0·25	52	0·02–0·01
	0·25	54	0·05–0·02
	0·1	67	>0·05
	0·1	>100	>0·05
m-Cresol	0·25	84	>0·05
	0·25	80	>0·05
	0·1	>100	>0·05
p-Cresol	0·25	52	0·05–0·02
	0·25	64	>0·05
	0·25	49	0·05–0·02
	0·1	59	0·01–0·001
	0·1	>100	>0·05
Thymol	0·25	55	0·02–0·01
	0·25	72	>0·05
	0·1	63	0·05–0·02
α-Naphthol	0·25	84	>0·05
β-Naphthol	0·25	94	>0·05
o-Hydroxybenzoic acid	0·25	74	>0·05
m-Hydroxybenzoic acid	0·25	88	>0·05
	0·25	89	>0·05
p-Hydroxybenzoic acid	0·25	81	>0·05
Saligenin	0·25	53	0·01–0·001
	0·25	44	0·02–0·01
	0·1	>100	>0·05

none of the compounds produced more than 15–30 min. slight lethargy, or very mild shivering detectable only by palpation.

Experiments designed to investigate the effect of 2 hr. deep narcosis and shock upon thyroid iodine uptake were carried out.

The iodine uptake was measured by the same method used for testing the effect of the phenol derivatives. The results are presented in Table 2. It will be seen that nembutal produced no significant lowering of iodine uptake and ether anaesthesia a reduction to 60% of that of the controls. Two groups of rats were shocked by the combined injections of magnesium sulphate and adenosinetriphosphate, as described by Green & Stoner (1944). The magnesium sulphate (75 mg./100 g. body weight) was given subcutaneously. The adenosinetriphosphate (ATP) was given intraperitoneally in

Table 4. *Effect on thyroid iodine uptake of resorcinol and certain derivatives*

(All experiments were in groups of four rats unless otherwise stated.)

Name of compound	m-moles/rat	Thyroid uptake of ^{131}I (%)	*P*
Resorcinol	0·25	14	Range of 8–23% (mean of 9 exps.)
	0·125	23	0·01–0·001 (2 rats)
	0·1	24	Range of 11–45% (mean of 11 exps.)
	0·06	40	0·05–0·02
Orcinol (5-methylresorcinol)	0·25	15	<0·001
	0·1	23	0·01–0·001
	0·06	46	0·05–0·02
2-Methylresorcinol	0·25	11	<0·001 (3 rats)
	0·1	11	<0·001
	0·06	54	0·05–0·02
4-Chlororesorcinol	0·25	13	0·01–0·001 (2 rats)
	0·1	25	<0·001
	0·06	41	0·02–0·01
4-Hexylresorcinol	0·25	51	0·01–0·001
	0·25	22	0·01–0·001
	0·1	80	>0·05
Resorcinol monacetate	0·25	13	<0·001
	0·1	16	0·01–0·001
	0·06	59	>0·05
Resorcinol diacetate	0·25	13	<0·001
	0·1	23	0·05–0·02
Resorcinol monomethyl ether	0·25	32	0·02–0·01
	0·25	71	>0·05
	0·1	64	0·05–0·02
Resorcinol dimethyl ether	0·25	97	>0·05
2:4-Dihydroxybenzoic acid	0·25	·23	<0·001
	0·25	19	<0·001
	0·1	63	>0·05
	0·1	51	0·05–0·02
	0·1	24	<0·001
2:4-Dihydroxybenzaldehyde	0·25	13	0·05–0·02
Naphthylresorcinol (1:3-Dihydroxynaphthalene)	0·25	18	<0·001
	0·25	16	<0·001
	0·25	9	<0·001
	0·1	20	<0·001

divided doses totalling 20 mg./100 g. rat in Exp. 33 and 82·5 mg./100 g. rat in Exp. 35. The former produced a severe toxic state lasting 1 hr., the latter a severe toxic state lasting the full 2 hr. supervening between commencement of the experiment and killing of the animals. The rats were cold and extremely lethargic. The thyroid iodine uptake was significantly lowered in both groups: to 58% in Exp. 33 and 40·5% in Exp. 35; but by no means so effectively as the reduction to 14% produced by the non-toxic (0·1 m-mole) dose of resorcinol in Exp. 34. It would appear from these results that deep narcosis or a non-specific toxic state are very unlikely to produce more than two- to threefold lowering of thyroid iodine uptake within the conditions of our experimental method. However, it is probable that the marked narcotic effects observed with the dihydroxynaphthalenes contributed significantly to the lowered thyroid uptake found.

DISCUSSION

Tables 3–5 show that all the most active compounds except 2:7-dihydroxynaphthalene, are, or may be considered as, resorcinol derivatives. Resorcinol itself is strongly active: its isomers, quinol and catechol, are inactive. The activity of the resorcinol and hydroxyquinol acetates is probably due to hydrolysis in the body. The non-activity of the methyl ethers supports this conclusion, since one would not expect them to be so hydrolysed. The substitution of a chlorine atom in the 4-position or

Table 5. *Effect on thyroid iodine uptake of polyhydric phenols and certain derivatives*

Name of compound	m-moles/rat	Thyroid uptake of ^{131}I (%)	*P*
Resorcinol	0·25	14	Range of 8–23% (mean of 9 exps.)
	0·125	23	0·01–0·001 (2 rats)
	0·1	24	Range of 11–45% (mean of 11 exps.)
	0·06	40	0·05–0·02
Catechol	0·25	53	>0·05 (3 rats)
	0·125	>100	>0·05 (6 rats)
	0·1	>100	>0·05
Quinol	0·25	61	0·05–0·02
	0·25	61	0·05–0·02
	0·25	70	>0·05
	0·125	>100	>0·05
	0·1	>100	>0·05
1:3-Dihydroxynaphthalene	0·25	18	<0·001
	0·25	16	<0·001
	0·25	9	<0·001
	0·1	20	<0·001
2:3-Dihydroxynaphthalene	0·25	17	<0·001
	0·25	25	0·01–0·001
	0·25	56	0·01–0·001
	0·1	43	<0·001
	0·1	58	0·01–0·001
	0·1	72	>0·05
1:5-Dihydroxynaphthalene	0·25	40	0·01–0·001
	0·25	53	0·01–0·001
	0·1	70	>0·05
2:7-Dihydroxynaphthalene	0·25	18	0·01–0·001
	0·25	18	<0·001
	0·1	17	<0·001
Pyrogallol	0·25	14	<0·001
	0·25	48	0·02–0·01
	0·1	83	>0·05
	0·1	67	0·05–0·02
	0·1	54	0·01–0·001
	0·1	56	0·02–0·01
Phloroglucinol	0·25	16	<0·001
	0·25	25	<0·001
	0·1	11	<0·001
	0·1	15	<0·001
	0·1	33	<0·001
Hydroxyquinol	0·25	17	<0·001
	0·1	22	<0·001
	0·1	15	0·05–0·02
Hydroxyquinol triacetate	0·1	28	0·05–0·02
2:4-Dihydroxybenzoic acid	0·25	23	0·01–0·001
	0·1	63	>0·05
2:5-Dihydroxybenzoic acid	0·25	>100	>0·05
	0·1	91	>0·05
Gallic acid	0·25	77	>0·05
	0·1	93	>0·05
	0·1	72	>0·05

a methyl group in the 2- or 5-position does not diminish the antithyroid activity of resorcinol, nor does the presence of a carboxyl group: 2:4-dihydroxybenzoic acid was comparable to resorcinol in its activity, whilst its 2:5-isomer proved inactive.

The interpretation of the results becomes more debatable when a third hydroxyl group is present as a substituent in the ring. The strongly marked activity of phloroglucinol is to be expected, since, for our purpose, it can be regarded as a resorcinol derivative. Its isomer pyrogallol, which may be regarded as a hydroxy derivative of either catechol

Table 6. *Summary of antithyroid effects of compounds tested*

Dose (m-mole)	Group 1. Active. Uptake 11–34% of controls	Group 2. Slightly active. Uptake 40–58% of controls	Group 3. Inactive. Uptake over 63% of controls
0·25	2-Methylresorcinol Resorcinol monacetate Resorcinol diacetate 4-Chlororesorcinol 2:4-Dihydroxybenzaldehyde Resorcinol (1:3-dihydroxybenzene) 1:3-Dihydroxynaphthalene Orcinol (5-methylresorcinol) Hydroxyquinol (1:3:4-trihydroxybenzene) 2:7-Dihydroxynaphthalene Phloroglucinol (1:3:5-trihydroxybenzene) 2:4-Dihydroxybenzoic acid Pyrogallol (1:2:3-trihydroxybenzene) 4-Hexylresorcinol	2:3-Dihydroxynaphthalene Phenol 1:5-Dihydroxynaphthalene Saligenin (*o*-hydroxybenzyl alcohol) Resorcinol monomethyl ether *o*-Cresol Catechol (1:2-dihydroxybenzene) *p*-Cresol	Quinol (1:4-dihydroxybenzene) Thymol (5-methyl-2-*iso*propylphenol) *o*-Hydroxybenzoic acid Gallic acid *p*-Hydroxybenzoic acid *m*-Cresol α-Naphthol *m*-Hydroxybenzoic acid β-Naphthol Resorcinol dimethyl ether 2:5-Dihydroxybenzoic acid
0·1	2-Methylresorcinol Resorcinol monacetate 2:7-Dihydroxynaphthalene Hydroxyquinol 1:3-Dihydroxynaphthalene Phloroglucinol Resorcinol diacetate Orcinol Resorcinol 4-Chlororesorcinol Hydroxyquinol triacetate 2:4-Dihydroxybenzaldehyde	2:4-Dihydroxybenzoic acid 2:3-Dihydroxynaphthalene	Thymol Resorcinol monomethyl ether Pyrogallol 1:5-Dihydroxynaphthalene *p*-Cresol 4-Hexylresorcinol Gallic acid 2:5-Dihydroxybenzoic acid Saligenin Phenol *m*-Cresol Catechol Quinol

A few compounds found inactive at the 0·25 m-mole concentration were, for that reason, not tested at the 0·1 m-mole concentration.

or resorcinol, is markedly less antithyroid than resorcinol. On the other hand, the antithyroid action of hydroxyquinol is strongly marked, indicating that it acts as a resorcinol derivative. The total inactivity of gallic acid helps to confirm the contrast found between pyrogallol and phloroglucinol.

In order to summarize the findings, we expressed each mean count rate on each experimental day as a percentage of the mean control figure on that day. When more than one test had been made of a given compound in the same concentration the percentages found on different occasions were averaged. These final percentages (Table 6) fell into three groups. Group 1 showed a reduction in iodine uptake to 11–34%, group 2 a reduction to 40–58%, and group 3 no significant reduction.

The compounds are arranged for each dose level and in each column in order of diminishing activity. Thus, with the 0·25 m-mole dose, 2-methylresorcinol produced an 11% and 4-hexylresorcinol a 34% uptake. This method of treatment of the results is of value as a means of expressing definite trends, though it does not give an accurate presentation.

Those compounds which showed slight activity at 0·25 m-mole and were inactive at 0·1 m-mole have been regarded as not proven to be antithyroid. Of the two compounds which showed slight activity at the 0·1 m-mole dose level, 2:4-dihydroxybenzoic acid is a resorcinol derivative and exhibited marked antithyroid activity at the higher concentration. The other compound, 2:3-dihydroxynaphthalene, showed a feeble antithyroid activity which was only slightly enhanced at the higher concentration.

The results strongly favour the conclusion that this form of antithyroid action arises from the presence of two hydroxyl groups meta-substituted to each other in a benzene ring. The 2:7-dihydroxynaphthalene forms the only major exception to this conclusion. 4-Hexylresorcinol was inactive at the lower concentration.

Possible modes of action of resorcinol derivatives. Evidence was put forward previously (Doniach & Fraser, 1950) that resorcinol interfered in the thyroid with the organic binding of iodide and did not lower the concentration of inorganic iodide. In this it simulates the action of thiouracil rather than that of thiocyanate.

In the opinion of Pitt-Rivers (1950) 'the antithyroid action of any compound may be expressed as a function of its reducing power and preferential reactivity with iodine, which inhibits the formation of thyroxine...'. It may be doubted, however, whether this hypothesis can fully account for all the facts established in the present investigation, since, among the compounds which we tested, there is no correlation between the antithyroid action and reducing power. Aromatic compounds containing hydroxyl groups oriented ortho or para with respect to each other (such as catechol, quinol, pyrogallol and hydroxyquinol) are stronger reducing agents *in vitro* than the meta isomers, yet in these experiments most of them have shown no distinctive antithyroid effect.

Differences in antithyroid potency may reflect differential concentration in the thyroid rather than differences in avidity for iodine. Garton & Williams (1949) studied the excretion of quinol, catechol and resorcinol in rabbits, and found that the first two compounds are almost entirely conjugated when excreted, only 2% of the catechol and a trace of quinol being isolated free in the urine. On the other hand, as much as 11–12% of resorcinol was excreted free. This quantitative difference may mean that the resorcinol-treated rats might have a higher blood level of the phenol than rats treated with catechol or quinol.

Dr W. E. Knox, of the National Institute for Medical Research, Mill Hill, brought to our notice a significant correlation between our results and those of Elliott (1932) who catalysed the oxidation of a series of phenols by hydrogen peroxide and milk peroxidase. The compounds tested included phenol, the cresols, catechol, quinol, resorcinol, pyrogallol, gallic acid and β-naphthol. Resorcinol alone was not oxidized by milk peroxidase, the activity of which it inhibited.

Elliott's findings, taken together with Dempsey's (1944) histochemical demonstration of a thyroid peroxidase and the demonstration of such a peroxidase *in vivo* by De Robertis & Grasso (1946), are compatible with the existence of a specific peroxidase of the thyroid gland, similar to milk peroxidase in that it is inhibited by resorcinol, but not by other phenols.

SUMMARY

1. The effect of a representative selection of phenolic substances on thyroid iodine uptake in the rat was studied, using radioactive iodine.

2. With one exception, all the compounds found to be strongly antithyroid could be regarded as derivatives of resorcinol.

3. Possible modes of action of these derivatives on the thyroid are discussed.

It is a pleasure to acknowledge our indebtedness to Dr D. A. Mitchison and Mr J. W. Boag, for statistical assistance, and to Prof. R. T. Williams for advice on the purification of hydroxyquinol.

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The Activity of Enzymic and Acidic Digests of Insulin

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In attempting to determine the nature of insulin and to obtain an active grouping or adjunct from it, many degradations have been carried out using proteolytic enzymes and acids. These studies were summarized by Hill & Howitt (1936) and by Jensen (1938). Proteolytic enzymes were favoured for this work as the mild conditions of their use and their specific action allowed more chance of preserving an active grouping.

The older work is unanimous in declaring that the activity of insulin is readily destroyed by impure pepsin, trypsin and papain, but in general only whole digests were examined for activity. Fisher & Scott (1934), however, examined the activity of the trichloroacetic acid precipitates of a crystalline

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peptic digest, and Banner, Derenvik & Thomas (1942) found that pancreatin and pepsin digests gave ultrafiltrates without any hypoglycaemic activity. No work had been described in which crystalline trypsin and chymotrypsin were used. However, since then, Butler, Dodds, Phillips & Stephen (1948*a*, *b*, 1949) have described the action of crystalline chymotrypsin, trypsin and pepsin on crystalline insulin.

This paper gives the results of tests of hypoglycaemic activity on fractions of peptic, chymotryptic and tryptic digests of insulin, together with some results for acid hydrolyses and the effect of miscellaneous agents on insulin activity.

EXPERIMENTAL

Insulin and crystalline enzymes. The same materials were used as in the previous work (Butler *et al.* 1948*a*, *b*, 1949). The crystalline pepsin used there and here was porcine, the insulin and chymotrypsin bovine. The crystalline insulin assayed 22 units/mg. (Boots Pure Drug Co. Ltd.).

Digestions. Unless otherwise specified, the peptic and chymotryptic digestions were carried out under the same conditions as in the previous papers, generally using 5 mg. insulin/ml. and approximately 4×10^{-4} units of enzyme/ml. The temperature was 25°. The tryptic digest was prepared with a sample of specially purified trypsin (bovine) kindly given by Dr J. H. Northrop and Dr M. Kunitz and used also in previous work (Butler *et al.* 1949).

Insulin activity. The samples tested were aqueous solutions and occasionally suspensions. The largest injection given was 4 ml. All but a few of the injections (noted in the text) were subcutaneous into rabbits of weight 1·5–2·5 kg. The rabbits were starved for 18 hr. before injection and blood-sugar samples were taken from the marginal ear vein immediately prior to and 1, 2 and 3 hr. after injection. All the rabbits used gave good and similar blood-sugar curves with 1 unit of a standard insulin solution, and as little as 0·125 unit consistently gave a depression of the blood-sugar level to about 10% (maximum) below the starving level. The blood sugar was estimated in 0·2 ml. samples by the method of Folin & Wu (1929).

Owing to the large number of samples it was desired to test, most of the estimates of potency were made with single rabbits. In view of the results obtained it is not felt that this approximate method has affected the conclusions. Moreover, some of the results are supported by quantitative assays kindly carried out by Boots Pure Drug Co. Ltd. and reported previously (Butler *et al.* 1948*a*, 1949).

Nitrogen. Nitrogen was determined by the micro-Kjeldahl modification of Ma & Zuazaga (1942). Non-protein nitrogen (N.P.N.) was generally determined by adding trichloroacetic acid to the digest to 0·25 N final concentration and measuring the nitrogen in a sample of the filtrate or supernatant after centrifugation.

RESULTS

Chymotryptic digests

The degradation of insulin by chymotrypsin (Butler *et al.* 1948*a*) gives three fractions: (*a*) undigested insulin, largely precipitable by bringing the digest to pH 5·5, the isoelectric point of insulin; (*b*) the insulin 'core', precipitable in 0·25 N-trichloroacetic acid (for a further description of the properties of this material, see Butler, Phillips, Stephen & Creeth, 1950); and (*c*) a mixture of small peptides, averaging tetra-pentapeptides, which are not precipitated in 0·25 N-trichloroacetic acid (TCA). Fractions (*a*) and (*b*) come down together when TCA is added to the whole digest. Estimates of the hypoglycaemic activity of the whole digest and the three fractions are given in Tables 1–4 and in Fig. 1.

Table 1. *Activity of whole chymotryptic digests of insulin*

(Digestion at pH 8·5 and 25°.)

Non-protein nitrogen (%)	Dose in terms of original insulin units	Approximate activity found (units)	Approximate activity remaining (%)
Start (2 min.)	1·2	0·5	50
—	1·9	>1	>50
30·4	2·4	1	40
40·8	4·8	0·5–1	10–20
47·5	14	0·5	3
—	36	1·5	4
—	144	>1 (convulsed)	>1

Table 2. *Activity of whole chymotryptic digests of insulin prepared at different* pH *values*

pH of digest	Non-protein nitrogen (%)	Dose in terms of original insulin units	Approximate activity found (units)	Approximate activity remaining (%)
7·6	32	2·4	0·3–0·5	12–20
7·7	69	19	1	5
8·4	73	19	1	5
9·8	65	19	Nil	0
10·2	65	19	Nil	0
10·5	40	2·4	0·5	20

Table 3. *Activity of the insulin core (digests C 28 and C 30)*

(The cores were prepared by TCA precipitation at 0·25 N final concentration after removal of the precipitate which forms in the digest at pH 5·5.)

Non-protein nitrogen (%)	Estimated percentage of core	Dose (mg. N)	Activity found/mg. N (units)	Activity of core compared with insulin = 100
22·4	23	0·20	5	3
30·4	30	0·10	5	3
29	37	0·30	1·7	1
40·8	42	0·13	3·7	2
42	44	0·30	1·7	1
47·5	52	0·136	Nil	0

Whole digests. Tables 1 and 2 show that the destruction of insulin activity increases with the formation of N.P.N. and at all digestion pH values tried. No chymotryptic digests have yet been made at pH values acid to 7, as insulin is too insoluble below this pH.

Precipitates obtained at pH 5·5. The correlation between the remaining activity of a whole chymotryptic digest at different times and the amount of precipitate obtainable from it by adjusting samples to pH 5·5 is shown in Fig. 1.

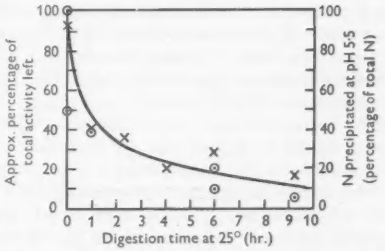

Fig. 1. Correlation between remaining activity of chymotryptic digest of insulin (⊙) and the amount of precipitate obtainable from it at pH 5·5 (×).

With digest C 29, two precipitates were prepared when the N.P.N. values of the digest were 15·4 and 24·4 % of the total nitrogen respectively. Injection of an amount containing 0·0062 mg. N (the nitrogen equivalent of 1 unit of insulin activity in the form of insulin of 25 units/mg.), showed that the earlier precipitate had the full activity and the later one about half the activity of pure insulin. These results show that although the pH 5·5 precipitate contains inactive material, it approximates to the amount expected from the remaining activity of the digest in terms of intact insulin. This suggests that all the activity remaining in a digest is due to the undigested insulin still present. That this is so is shown by the lack of activity in the other two fractions of the digests.

Insulin 'core'. It can be shown that the 1–3 % of activity, compared with insulin, in the insulin 'core' (Table 3), is due to associated insulin which escapes precipitation at pH values near 5·5. Thus an undigested insulin solution adjusted to pH 5·7, and centrifuged and filtered after 4 hr. at 20°, gave a filtrate containing about 1 unit of activity from each 34 units of insulin originally taken, so that approximately 3 % escaped precipitation. The filtrate actually had 0·5 unit of activity/ml. Lens (1948) found that the solubility of pure insulin in pH 5·5, 0·1 M-acetate buffer is 30 mg./l., corresponding to 0·75 unit of activity/ml.

Table 4. *Activity of the small TCA-soluble peptides from chymotryptic digests of insulin*

Non-protein nitrogen when sample was taken (%)	Dose (mg. N)	Approximate activity found (units)	Activity of the same amount of insulin N (units)
15·4	0·056	Nil*	9
24·4	0·10	Nil*	16
30·4	0·063	Nil*	10
36·3	0·025	Nil*	4
—	0·123	Nil	20
40·8	0·256	Nil	41
47·5	0·274	Nil	44

* These results showed hyperglycaemic activity, due to adverse reaction to injection. Bulked samples tested later showed no effect at all (see text).

This insulin remaining in solution would be precipitated by TCA. The efficiency of this precipitant was shown by injecting the neutralized filtrate from 71 units of insulin, after precipitation by 0·25 N final concentration of TCA. The maximum blood-sugar fall was 18 % of the starving level, corresponding to 0·25 unit of insulin remaining in solution. If 5·8 mg. of the peptides derived from insulin by chymotryptic digestion were also present, a maximum blood-sugar fall of 10 % occurred. Under the conditions used in this work, one unit of insulin invariably gave a maximum blood-sugar fall of 40–60 % of the starving level.

Thus the core activity is actually due to traces of insulin in it. In an earlier paper (Butler *et al.* 1948*a*), it was shown by quantitative assays, kindly carried out by Boots Pure Drug Co. Ltd., that TCA pre-

cipitates from whole digests (i.e. containing core and undigested insulin), had an activity equivalent to that expected from the amount of insulin still present, as measured by the amount of precipitate obtainable from the digest samples at pH 5·4.

Small peptides. The injection of up to 16 mg. of peptides (those samples which had shown hyperglycaemia in Table 4 being bulked together and concentrated) had no effect at all on the blood sugar. The largest dose given was derived from the degradation of 750 units of insulin.

Intravenous injection of insulin causes a more rapid effect on the blood sugar than subcutaneous injection, and this route thus allows less time for adverse factors such as tissue enzymes or inhibitors to prevent the display of hypoglycaemic activity. However, the intravenous injection of 54–58 mg. of the peptides from a chymotryptic digest of insulin (C 26, which had digested to a value of 63 % N.P.N.) had virtually no hypoglycaemic effect, one case showing a maximum fall of 18 % of the starving blood-sugar level and other tests showing only considerable hyperglycaemia.

In preparing peptides for injection, the excess TCA used for precipitating the core was removed either by repeated extraction with ether or merely by neutralizing the TCA filtrate. Neither procedure had any effect in itself on the blood-sugar estimations, since intermittent shaking of an insulin solution with ether for 2 hr. had no effect on its potency and the injection of 1 ml. of M-sodium trichloroacetate (183 mg.) produced only a 10 % maximum fall of blood-sugar level below the starving level, and moreover did not prevent 1 unit of insulin from exerting its full effect when admixed with such an amount.

Peptic digests

Like the chymotryptic digests, peptic digests yield three fractions: (*a*) The remaining intact insulin and other insulin-like material, all precipitated at pH 5·5. (*b*) A large fragment corresponding to the chymotryptic 'core' is precipitated either by heating the neutralized digest, by freezing and thawing or by the addition of TCA to N final concentration. (*c*) The small peptides soluble in N-TCA. The N.P.N. of peptic digests in 0·25 N-TCA rapidly reaches 100 %, but in N-TCA does not exceed 50 % of the total nitrogen during the digestion.

Tables 5–7 give the activities of whole digests and fractions (*a*) and (*c*).

Precipitates obtained at pH 5·5. The isoelectric precipitates had activities lower than insulin, in accordance with the finding (Butler *et al.* 1948*b*) that adjusting an insulin-free peptic digest to a pH between 4 and 7 resulted in the slow precipitation of insoluble material.

Peptic 'core' of insulin. Two 5 mg. samples of the precipitate formed by freezing digest P 6 had no hypoglycaemic activity when injected subcutaneously into two rabbits. This amount was derived from the digestion of 590 units of insulin, and the same weight of insulin would have had 110 units of activity. Similarly, 4·9 mg. of the precipitate formed by heating digest P 9 (N.P.N. = 100 %) had no influence on the blood sugar.

Dr K. Pedersen of the Physico-Chemical Institute, Upsala, kindly examined this P 9 precipitate. The ultracentrifuge showed the presence of polydisperse material and a slow sedimenting component giving $s_{20} = 1{\cdot}1S$ (average). The best of two diffusion measurements gave the constants $D_A = 20{\cdot}5$ and

Table 5. *Activity of whole peptic digests of insulin*

(The digests were prepared in 0·05 N-HCl.)

Digest no.	N soluble in 0·25 N-TCA (%)	Dose in terms of original insulin units	Approximate activity found (units)	Approximate activity remaining (%)
30/5	7·8	1·3	1	80–100
P 12	16	1·6	1	60
P 13	22	2·0	1	50
P 12	35	3·0	0·5	20
P 13	39	3·0	0·5–1	20–40
30/5	83	1·3	0·25*	20
P 12	100	81·0	Nil	0

* Maximum fall of blood sugar 21 % of original (starving) level.

Table 6. *Activity of* pH 5·5 *precipitates from a peptic digest*

Total N precipitated at pH 5·5 (%)	Dose (mg. N)	Activity of the same amount of insulin N (units)	Approximate activity found (units)	Approximate activity compared with insulin = 100
75	0·0065	1	0·5	50
49	0·0065	1	0·33	33

Table 7. *Activity of the small peptides soluble in* 1N-*TCA from peptic digests of insulin*

Digest no.	N soluble in N-TCA (%)	Dose (mg. N)	Activity of the same amount of insulin N (units)	Approximate activity found (units)
P12 (i)	9	0·026	4·3	Nil
P12 (ii)	16	0·035	5·7	Nil
P12 (i) + (ii)	—	0·113	18	Nil
P14	5	0·054	9	Nil
P14	9	0·103	17	Nil
P14	13·6	0·036	6	Nil*

* Maximum fall of blood sugar was 16% of the starving level.

$D_M = 21{\cdot}4$. Combining these, Dr Pedersen concluded that the average molecular weight of the low molecular weight material in P 9 was 4000–5000.

Small peptides. Other digests made at pH values from 1·3 to 2·9 also gave peptide fractions which were inactive. Attempts were made to digest insulin at more alkaline pH values with pepsin, but in 0·1M-citrate-0·1N-hydrochloric acid buffer at pH 3·8, the insulin was almost completely insoluble. Solutions of insulin could be prepared in formate-formic acid at pH 4–4·5, but under these conditions the pepsin and insulin co-precipitated immediately when mixed (Phillips, unpublished).

Intravenous injections. The intravenous injection of a sample of the peptides from a peptic digest (0·15 mg. N, equivalent to 24 units if all insulin N) gave a slight erratic hyperglycaemia, but no fall of blood sugar over the course of 1·5 hr., when measured at 20 min. intervals.

Tryptic digests

The activity of whole digests has been discussed in two previous papers (Butler *et al.* 1948*a*, 1949). Insulin (5 mg./ml.) was digested with 5×10^{-4} units of purified crystalline trypsin/ml. of digest at pH 8·5 and 25°. The N.P.N. (as measured by adding 1 ml. of N-TCA to 3 ml. of the digest) was 7·8 and 15·9% of the total nitrogen after 22 and 45 hr. digestion respectively. After removal of the excess TCA with ether the peptides were tested and none depressed the rabbit blood sugar more than 9% of the starving level. Doses up to 3·4 and 7·2 mg. from the 22 and 45 hr. digests respectively were given subcutaneously. The approximate activities of the whole digest after 22 and 45 hr. digestion were 60 and 30% of the original respectively, which contrasts with the value of about 60% found previously (Butler *et al.* 1949) after 48 hr. digestion. In the present case, however, the enzyme concentration was 3·7 times greater.

The effect of other agents on the activity of insulin

The lack of effect of ether on the potency of an insulin solution has already been mentioned, but in the course of the work the effect of other agents was also tried.

Trichloroacetic acid. An insulin solution (5 ml.) containing 2 units/ml. (0·09 mg./ml.) was precipitated by 5 ml. of 0·3N-TCA, centrifuged, and the precipitate redissolved in 5 ml. of water. Injection of 0·5 ml. of this solution gave a maximum fall of 40% in the blood-sugar level compared with a 35% maximum fall for 0·5 ml. of the original solution with the same rabbit. The potency was thus unaffected by the precipitant and the high efficiency of TCA in precipitating such a weak protein solution was demonstrated. This is supported by the finding already reported that the TCA filtrates from 71 units of insulin had no hypoglycaemic activity.

Urea. By the addition of urea, solutions of insulin can be made at pH 4–7, where it is normally insoluble. As a preliminary to experiments on the digestion of insulin by enzymes between these pH values it was essential to know whether urea would inactivate the insulin.

Clear solutions of insulin were made in 5M-urea at various pH values from 3·2 to 10·1 and kept at 25° for 22 hr. No loss of activity was shown by the pH 3·2, 6·4 and 10·1 samples, and about 50% loss at pH 5·0 and 8·2. Further tests showed no loss after 50 hr. at pH 4·5 and 6·4. These variations must be attributed to the use of an approximate method for estimating the potency, but together implied that 5M-urea could be used safely for a sufficiently long time for enzymic hydrolysis to take place. In a trial experiment it was found that chymotrypsin would digest insulin in 5M-urea at pH 8, and that all the activity had been destroyed by the time the non-protein nitrogen had reached 76%.

It was further found that solutions could be achieved at pH 4·5 and 6 in only 2M-urea, but even at this concentration urea interferes slightly with the precipitation of insulin by TCA. Solutions of 39 and 49 units of insulin were precipitated in the presence of 0·94M- and 0·69M-urea and 0·22N- and 0·17N-TCA respectively (all concentrations refer to final concentrations after the addition of TCA). Only 1% of the insulin activity remained in solution. Even this may be due to the insufficient duration (about 0·5 hr.) of standing before centrifuging the suspension. An injection of 4 ml. of 0·5M-urea,

more than was given in the injections above, caused a 9 % rise in the starving blood-sugar level.

Acid hydrolysis of insulin. The activity of acid hydrolysates of insulin has not been reported in detail. Hot 0·1N-hydrochloric acid forms a reversibly inactive precipitate (Jensen & Evans, 1932; Du Vigneaud, Sifferd & Sealock, 1933). The use of concentrated acids at low temperatures has some promise in this work in that Sanger (1948*b*, 1949) has been able to isolate peptides up to pentapeptide in size by such hydrolyses of insulin.

For potency trials a high ratio of insulin to acid is preferable so that the samples tested can be considerably diluted before injection, and will then contain little acid or salt to cause unfavourable reactions in the rabbit. Dilution is also necessary because a high hydrochloric acid concentration competes with the TCA for the insulin and precipitation is incomplete. In 7·7N-hydrochloric acid at 25°, the N.P.N. values were 60, 77 and 100 % after 0·8, 1·8 and 6 days respectively.

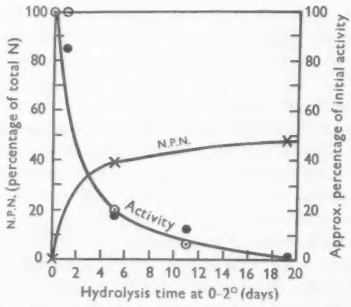

Fig. 2. The formation of N.P.N. (×) (N soluble in 0·25N-trichloroacetic acid) and the activity of insulin digests (●) and of the TCA precipitates obtainable from them (⊙), when insulin is digested in 8N-HCl at 0–2°.

Except for the initial sample none of the whole digests (injecting the equivalent of 8 units of insulin), nor the non-protein fractions (injecting the filtrate from 40 to 50 units of insulin) had any hypoglycaemic activity. The TCA precipitate from 66 units of insulin after 19 hr. hydrolysis, however, had a total activity of about 1 unit, though the subsequent TCA precipitates had none.

On the other hand 0·8N-hydrochloric acid had little effect on insulin, the activity falling to about 50 % only after 6 days at 25°. None of the N.P.N. samples had any definite activity even when derived from 76 units of insulin.

If the hydrochloric acid concentration is much above 0·8N, insulin immediately forms an insoluble precipitate which is not redissolved until the acid concentration is about 6N, though the limit depends upon the concentration of insulin.

Experiments were made with 8N-hydrochloric acid at 0–2°. Fig. 2 shows the formation of N.P.N., and the activity of the whole hydrolysates and the TCA precipitates. The TCA filtrates prepared at various stages of hydrolysis were inactive even when derived from 100 units of insulin. The TCA precipitates, therefore, accounted for all the activity shown by the whole digest samples.

Diisopropyl fluorophosphonate (DFP). DFP has a unique inhibitory effect on chymotrypsin and trypsin (Jansen, Nutting, Jang & Balls, 1949), the former enzyme requiring only 1 mol. DFP/mol. of enzyme of mol.wt. 27,000 for complete inhibition. Such specific inhibition may well lead to the finding of the active grouping or the most important part of these enzymes, and a comparable finding with insulin would thus be valuable.

Insulin was incubated with DFP (4·6–47 mol./mol insulin of weight 12,000) at pH 8 for periods up to 46 hr. at 25° without affecting the potency of the insulin compared with controls. This is illustrated in Table 8. It was confirmed that the DFP retained

Table 8. *Effect of diisopropyl fluorophosphonate (DFP) on insulin at* pH 8 *and* 25°

Treatment of sample	Dose in terms of insulin	Maximum fall of blood sugar level* (%)
18 hr. DFP: insulin ratio = 4·6	1·2	51
As above, then 28 hr. with ratio = 47·0	1·2	50
19 hr. DFP: insulin ratio = 42·0	1·2	50
Control. No DFP	1·2	42
Control, 48 hr. pH 8. No DFP	1·2	55
Control, pH 8·1 buffer with DFP. No insulin	—	14

* As percentage of the starving level = 100 %.

at least 20 % of its initial activity after 24 hr. at pH 8 by testing the extent of inhibition of a chymotrypsin solution (Jansen *et al.* 1949). The pH value 8 was chosen for the tests on insulin as this is near the optimum for the inactivation of trypsin and chymotrypsin by DFP. Furthermore, no evidence of reaction of DFP with any of the individual amino-acids found in insulin could be obtained from paper chromatograms of the acids after incubation with DFP (pH initially 8, but later fell to about 3 owing to the spontaneous hydrolysis of the DFP).

This accords indirectly with the more recent finding of Jansen, Nutting, Jang & Balls (1950), that none of the individual amino-acids of chymotrypsinogen when added to intact chymotrypsin affected the degree of inhibition of that enzyme by DFP.

DISCUSSION

The finding that no product of the digestion of crystalline insulin by crystalline pepsin, chymotrypsin or trypsin has any hypoglycaemic activity

confirms the results on whole digests obtained by earlier workers using impure enzymes.

No fraction or combination of fractions from the digests had any hypoglycaemic activity in large doses, irrespective of the pH of digestion, and all the remaining activity of the digests could be ascribed to the intact insulin still present. This is in spite of the fact that the crystalline proteolytic enzymes give larger protein fragments than the crude enzymes, there being almost no liberation of free amino-acids in the former case (see, for example, Van Slyke, Dillon, MacFadyen & Hamilton, 1941; Winnick, 1943; Butler *et al.* 1948*a*, *b*).

In all degradations of insulin, except the removal of amide groups by boiling 0·1 N-hydrochloric acid by Jensen & Evans (1932), the activity is reduced, and in the work of Fisher & Scott (1934) with pepsin, mentioned above, and that of Lens (1949) with carboxypeptidase, the insulin was largely inactivated though very little TCA-soluble nitrogen was formed. The 70% loss of activity during the 45 hr. tryptic digestion reported above, for the formation of only 15·9% N.P.N., also comes into this category.

These inactivations, however, can be interpreted in two ways. Either the whole or only a special part of the molecule is essential for activity. It seems likely (Butler *et al.* 1950) that the core of insulin of mol.wt. 5000, revealed by chymotryptic action, lies at the 'amino' end of the peptide chains of insulin and that the accompanying inactivation is due to the removal of small peptides from the ends of the chains bearing the free α-carboxyl groups. This suggests, like Lens's work, that if only part of the molecule is active, then it lies outside the large core and probably at the carboxyl ends of the peptide chains. One must then assume that all the degradations so far carried out have attacked the essential part.

Even if the whole molecule is required, certain parts will be more intimately involved in the *in vivo* reactions of the hormone. Thus, investigations of insulin derivatives (Sanger, 1948*a*; Fraenkel-Conrat & Fraenkel-Conrat, 1950; Mommaerts & Neurath, 1950) have established the essential nature of the cystine disulphide groups and of some of the tyrosine and carboxyl groups. Nevertheless, although the insulin core formed by the action of chymotrypsin contains all these groupings, no activity was found. Similarly, the tyrosine-cystine dipeptides prepared by Harington & Pitt-Rivers (1944) showed nothing more than a transient slight hypoglycaemic effect of doubtful significance (Prof. F. G. Young & Dr M. P. Whitehouse, private communication). The absence of hypoglycaemic activity in doses of peptides as large as 58 mg., moreover, implies that there are also no fragments present sufficiently like the structure of the hypothetical active group to give even fractional activity. Moreover, the fact that the peptides do not prevent added intact insulin from displaying full activity shows that such 'analogues', if present, are not inhibitory to its action.

In spite of this lack of success with insulin and in degradative studies on the proteolytic enzymes themselves (Northrop, 1932; Northrop & Kunitz, 1932; Northrop, Kunitz & Herriott, 1948), the finding by Li (1948, 1949, 1950) and Cortis-Jones, Crooke, Henly, Morris & Morris (1950) that the activity of certain very highly purified pituitary hormones can be obtained in the form of quite small peptides, though it is uncertain if these were originally attached to the protein by conventional peptide bonds, means that the hypothesis of an active peptide grouping in insulin, attached to a larger protein mass, cannot yet be rejected.

SUMMARY

1. Crystalline chymotrypsin, trypsin and pepsin degrade crystalline insulin to give peptide fractions which show no hypoglycaemic activity.

2. Digestion by purified crystalline trypsin causes considerable loss of activity although little non-protein nitrogen is formed.

3. The remaining activity shown by the whole digests is precipitable at pH 5·5, and is probably due to intact insulin still present.

4. Variation of the pH of digestion affects the rate but fails to give active fractions.

5. Hydrolysis with 8 N- and 0·8 N-hydrochloric acid at low temperatures similarly destroys the activity, and the non-protein fractions formed during hydrolysis have no activity.

6. Ether, trichloroacetic acid, 5 M-urea and di*iso*propyl fluorophosphonate have no appreciable effect on the activity of insulin in periods of contact up to 24 hr.

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Hormonal Factors Influencing Liver Catalase Activity in Mice. Testicular and Adrenal Factors

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Greenstein and his associates have shown that the presence of a growing tumour in rats and mice is almost invariably associated with a marked depression in liver catalase activity (Greenstein, Jenrette & White, 1941; Greenstein & Andervont, 1942).

As far as the mechanism of the effect is concerned, evidence has recently been obtained suggesting that the tumour exerts its effect through the medium of some toxic material released into the circulation. Nakahara & Fukuoka (1949, 1950) claimed to have produced depressions of liver catalase activity in mice by the injection of ethanol-precipitated fractions obtained from human tumours. Adams (1950*a*, 1951) has shown that mouse tumours in general contain some factor or factors capable of markedly and rapidly influencing liver catalase activity. The way in which such factors could operate is obscure. Greenstein (1943) looked for a direct inhibitor of liver catalase in tumours without success, and finally concluded that in some way tumours must be interfering with the synthesis of catalase in the liver. The purpose of the present work is to examine the mechanisms which control liver catalase activity in the normal animal as a preliminary to further investigation into the effect of tumours on the system.

During the course of the earlier work (Adams, 1950*a*, 1951) a sex difference in normal catalase activity was observed in all three strains of mice used—males having a level some 30% higher than females. It seemed likely, therefore, that there was some hormonal influence on catalase level. A preliminary report (Adams, 1950*b*) has already appeared on the effects of castration, and castration followed by adrenalectomy, in males, and adrenalectomy and ovariectomy in females. Marked falls in liver catalase level were observed within 48 hr. after all these operations, except ovariectomy in females, where there was no significant change.

Begg & Reynolds (1950) have also recently reported a depression of liver catalase in rats 3 weeks after adrenalectomy.

EXPERIMENTAL

Animals. Young adult mice (weighing 25–30 g.) of a stock albino strain were used. The diet of the animals consisted of rat cubes (supplied commercially) and water *ad lib.*

Estimation of liver-catalase activity. Samples of whole liver homogenates were allowed to react with 0·025 M-H_2O_2 (A.R. quality) in 0·02 M-phosphate buffer (pH 6·8) at 0°, the reaction being stopped after 4 min. by the addition of H_2SO_4. The H_2O_2 remaining was estimated by titration with standard thiosulphate after the addition of excess KI. For details see Adams (1950*a*). Catalase activity is expressed in arbitrary units/mg. N. Nitrogen rather than tissue wet weight has been chosen as a standard of reference for the following reasons: (1) The spread on the catalase activity within groups of animals tends to be reduced. (2) Greenstein *et al.* (1941) and Weil-Malherbe & Schade (1948) used total N and protein N, respectively. (3) There is evidence from starvation studies in rats that the fall in liver catalase activity observed after some days is to some extent paralleled by a fall in liver nitrogen content (Miller, 1947). Reference to N, therefore, will tend to correct for changes in nutritional status. However, from the experimental data obtained, it may be stated that substitution of tissue wet weight for N would have had no effect on the pattern of the results about to be described.

Operative procedures. Castration was carried out through a single median abdominal incision, and adrenalectomy through a single median skin incision over the lumbar spine and two para-median incisions through the posterior muscular abdominal wall. Fat associated with the adrenal glands was removed with them. No special precautions were taken post-operatively with castrated animals, but adrenalectomized animals were maintained at a temperature of approximately 27° (81° F.) and given salt water (0·5%). In all cases where subsequent injections were given an interval of 48 hr. was allowed post-operatively. The absence of adrenal tissue in adrenalectomized animals was checked post mortem.

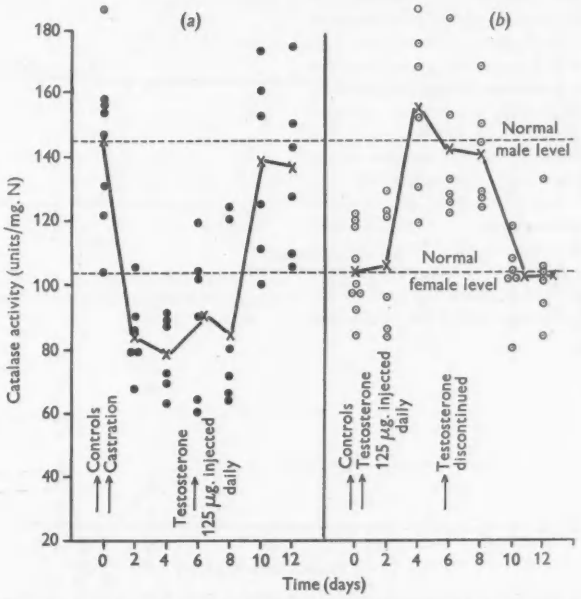

Fig. 1. *a*, Effect of castration and the subsequent injection of testosterone on the liver catalase activity of male albino mice. *b*, Effect of the injection of testosterone and its subsequent withdrawal on the liver catalase activity of female albino mice. ⊙, Individual mice; ×, the arithmetic mean values of the groups in this and subsequent figures.

Hormones and related substances

Testosterone. (Commercially supplied for implantation.)

Cortisone. (17-*Hydroxy*-11-*dehydrocorticosterone.*) Kindly provided by Prof. Wilson and the Medical Research Council.

Deoxycorticosterone glucoside. (Commercial aqueous solution.)

Progesterone. (Commercial suspension.)

All the above were used in 50% ethanol-water solution and injected subcutaneously. The maximum quantity of solution injected per day was 0·1 ml.

RESULTS

Effect of castration and testosterone administration

A number of male mice were castrated, and the liver catalase activity of small groups was determined every 2 days. Fig. 1 shows that 48 hr. after castration

the catalase activity had fallen considerably, remaining at the new low level on the 4th and 6th day. On the 6th post-operative day the remaining mice were each injected with 125 μg. of testosterone in 50% ethanol, the dose being repeated daily. The control animals received ethanol only. Two days later the catalase level was unaltered, but returned to normal after a further 2 days, and remained so. Fig. 1 also shows the effect on normal females of the injection of the same quantity of testosterone daily and its subsequent withdrawal. Four days from the start of the injections the liver catalase activity rose to approximately the normal male level. On the 6th day testosterone was discontinued, and 4 days later catalase activity returned to normal. It may also be seen from the graph that the castrated male level is near, but possibly slightly below, the normal female level. The daily administration of testosterone to normal male mice and its subsequent withdrawal after 6 days had little or no effect on catalase level (Table 1). Fig. 2 shows the effect on normal females of the daily injection of varying doses of testosterone over a period of 5 days. The liver catalase level rises sharply with dose up to about 40 μg./day, but there is little further rise up to 640 μg./day.

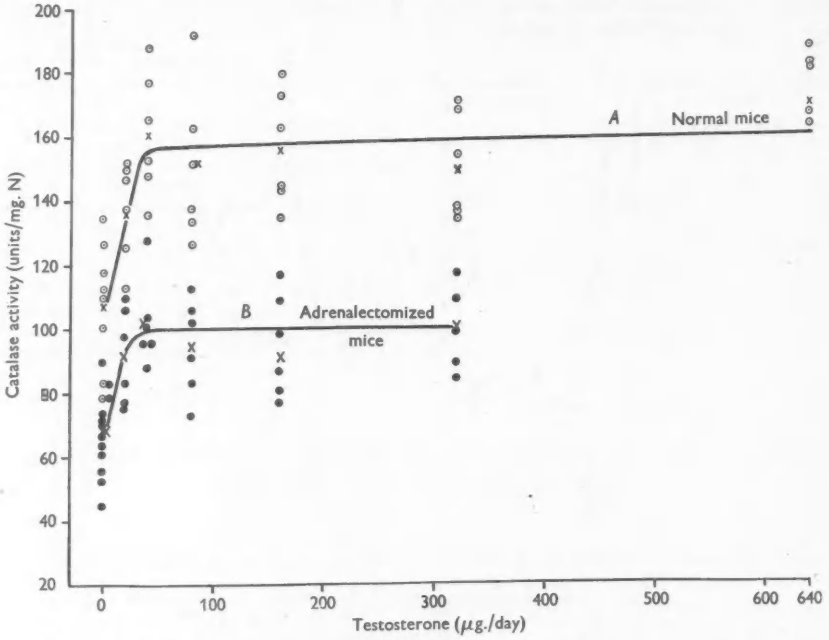

Fig. 2. Effect of the injection of varying amounts of testosterone daily for 5 days on the liver catalase activity of (*A*) normal female mice; (*B*) adrenalectomized female mice.

Effect of adrenalectomy and cortisone administration

As shown in Fig. 3 adrenalectomy of female mice resulted in a marked depression in liver catalase activity 48 hr. later, the level remaining low 4 and 6 days after operation. On the 6th day 70 μg. of cortisone in 50% ethanol was injected, the injections

Table 1. *Liver catalase level in male albino mice after the daily injection of testosterone*

Results are given in arbitrary units/mg. N as arithmetic means ± standard error of means. Six to eight animals/group.

Substance	Catalase level Normal controls	Dosage (μg./day)	Catalase level at varying times (days) after commencement of injection 2	4	6*	10	12
Testosterone	145 ± 7·0	125	152 ± 9·9	153 ± 7·3	150 ± 8·0	140 ± 6·6	136 ± 9·0

* Testosterone withdrawn.

being repeated twice daily. This was almost immediately effective, the catalase level returning to normal within 4 days. The effect of injecting adrenalectomized males with varying doses of cortisone daily for 5 days is shown in Fig. 4. A dose

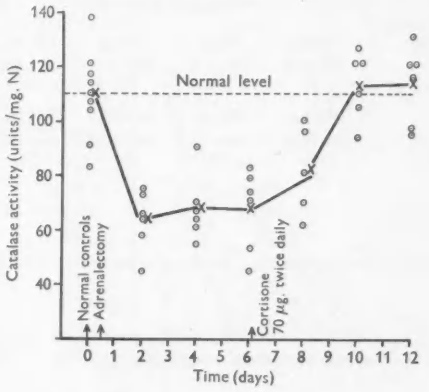

Fig. 3. Effect of adrenalectomy and the subsequent injection of cortisone on the liver catalase activity of female albino mice.

of 30 μg./day is sufficient to restore the normal level, there being little or no rise with higher doses. In these two experiments the control animals again received injections of ethanol only. However, in a separate experiment, results of which appear in Table 2, the injection over 5 days of up to 0·1 ml. ethanol/day was shown to have no effect on the catalase level of an adrenalectomized animal. The injection of ethanol into control animals was therefore discontinued in subsequent experiments.

Progesterone and deoxycorticosterone were also injected into adrenalectomized male mice over a period of 5 days. The results appear in Table 2. Progesterone in doses up to 240 μg./day gave no significant rise. Deoxycorticosterone in very high dosage produced a partial restoration of catalase activity. In this experiment only the adrenalectomized controls were given salt water.

To meet the possible criticism that the alterations in catalase level following adrenalectomy might be due to post-operative anorexia, a paired feeding experiment was carried out. As stated previously, adrenalectomized animals are maintained at a temperature of approximately 81° F. Since this alone would reduce calorie requirements it was considered best to maintain the paired-fed controls at this temperature also. For completeness, a group of controls at normal temperature (approx. 70° F.) with unrestricted food intake was included. The results appear in Table 3, and show that no significant difference was observed between the catalase levels of the paired-fed and normal controls. The food intake of the adrenalectomized animals rose steadily over 4 days. On the first day, the food intake of the controls which were subsequently to be paired fed, fell sharply compared with the control animals at the lower temperature.

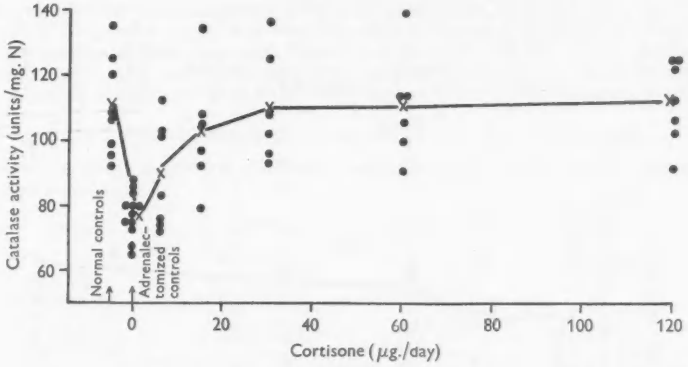

Fig. 4. Effect of the injection of varying doses of cortisone per day for 5 days on the liver catalase activity of adrenalectomized male albino mice.

Interrelation between adrenal and testicular factors

The results so far have shown that in adrenalectomized males and females the resulting lowered liver-catalase level is restored by cortisone. Testosterone restores the normal activity in castrated males, and elevates the normally lower female level to that of the male. It is therefore of interest to see whether testosterone and cortisone are capable of replacing one another. Testosterone in varying

Table 2. *Liver catalase level in adrenalectomized male albino mice after the injection of varying quantities of progesterone, deoxycorticosterone, or ethanol over a period of 5 days*

(Catalase levels in arbitrary units/mg. N expressed as arithmetic means ± standard errors of means. The figures in parentheses give the numbers of animals in the groups.)

Substance	Catalase level: Normal controls	Catalase level: Adrenalectomized controls	Catalase level 5 days after injection of varying quantities/day			
Progesterone	127 ± 6·3 (12)	75 ± 2·4 (8)	30 μg. 83 ± 6·6 (6)	60 μg. 94 ± 7·6 (6)	120 μg. 86 ± 6·7 (6)	240 μg. 82 ± 4·2 (6)
			100 μg.	200 μg.	400 μg.	1000 μg.
Deoxycorticosterone	113 ± 4·9 (12)	75 ± 4·1 (10)	80 ± 5·9 (6)	84 ± 4·1 (6)	94 ± 6·9* (6)	92 ± 5·5* (6)
			0·05 ml.		0·1 ml.	
Ethanol	141 ± 8·0 (8)	68 ± 4·5 (6)	68 ± 4·5 (6)		74 ± 4·0 (6)	

* These values are just significantly different from the adrenalectomized controls ($t = 2{\cdot}3$ and $2{\cdot}4$ respectively). ($t > 2$ for $P = 0{\cdot}05$.)

Table 3. *Liver catalase level in adrenalectomized male albino mice compared with that in paired-fed controls maintained at the same temperature* (81° F.), *and in controls at normal temperature having an unrestricted food intake*

(Results are given in arbitrary units/mg. N as arithmetic means ± standard error of means. Eight animals/group.)

	Catalase activity at varying times (days) after commencement				Food intake (g./mouse) at varying times (days) after commencement (averaged)				
	2	3	4	5	1	2	3	4	5
Adrenalectomized	79 ± 4·9	—	81 ± 3·9	—	2·2	3·2	3·6	4·9	—
Paired-fed controls	—	116 ± 6·0	—	127 ± 4·4	3·3	2·2	3·2	3·6	4·9
Normal mice	122 ± 8·2	—	128 ± 10·2	—	6·5	7·7	7·5	6·5	—

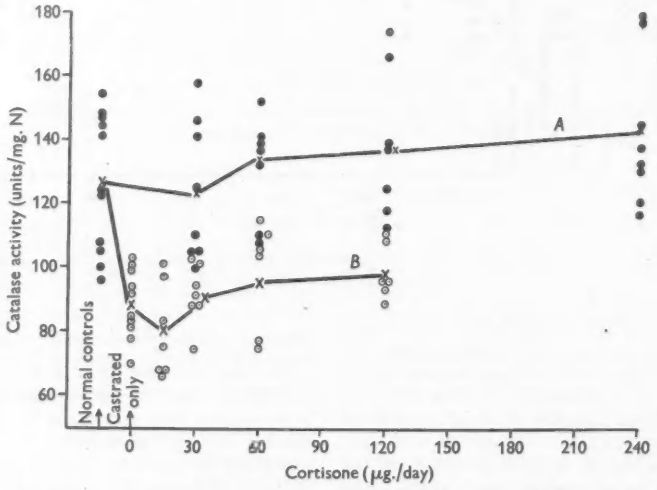

Fig. 5. Effect of the injection of varying doses of cortisone per day for 5 days on the liver catalase activity of (*A*) normal male albino mice; (*B*) castrated male albino mice.

dosage was injected into normal and adrenalectomized female mice. The results appear in Fig. 2. The two curves run approximately parallel to one another, showing that the effect on adrenalectomized mice is no greater than on normals, and therefore that, as far as catalase is concerned, testosterone will not replace adrenal secretion, although, as has already been shown, cortisone will do so. Nor has cortisone a greater effect on castrated than on normal males, as shown in Fig. 5. There is no sign, in the former, of a return to the normal catalase level. It appears, therefore, that cortisone and testosterone are exerting independent actions on liver catalase. There is, however, a complicating factor in male mice. Fig. 6*a* shows the effect on male liver catalase of castration followed by adrenalectomy some days later, and, in a corresponding experiment, of adrenalectomy followed by castration. Castration alone, and adrenalectomy alone, produced the usual depressions of activity at 48 hr., the latter greater than the former, and these were unchanged 48 hr. later. Adrenalectomy of the castrated animals resulted in a further fall, but the level was then only very little below that of the mice which had been adrenalectomized, but not castrated. Castration of the adrenalectomized animals produced little or no change in level, contrasting with the large fall obtained on castrating a normal animal. However, although castration after adrenalectomy produced little change, it is evident that the same ultimate level is reached independently of the order in which the operations are performed. A further large group of animals from the same batch were castrated, and adrenalectomized 48 hr. later. Catalase deter-

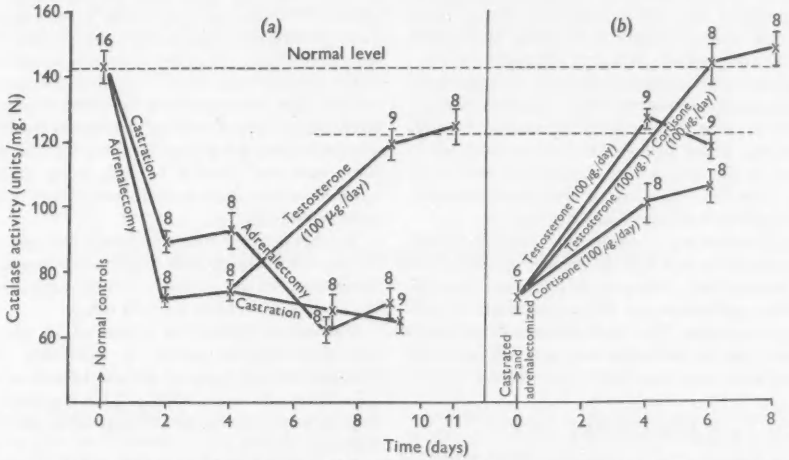

Fig. 6. *a*, Effect of castration or adrenalectomy followed by adrenalectomy or castration, respectively, on the liver catalase activity of male albino mice. Also shows the effect of testosterone injection on adrenalectomized males. *b*, Effect of the injection of testosterone, cortisone, and a mixture of both, on the catalase activity of castrated and adrenalectomized male albino mice. Results are given as arithmetic means (×) ±standard error of means. The number of animals in each group is given by the small figures at the head of the standard error limits.

Table 4. *Liver catalase levels in mice after castration and adrenalectomy*

(Results are given in arbitrary units/mg. N as arithmetic means±standard error of means. The figures in brackets give the number of animals in the groups.)

Controls	Castrated	Adrenalectomized	Castrated and adrenalectomized
	Males		
*145±5·3 (16)	91±2·8 (16)	74±2·4 (16)	68±1·8 (36)
141±80 (8)	—	68±4·5 (6)	—
145±7·9 (9)	85±4·1 (18)	—	—
110±5·4 (8)	—	77±1·9 (10)	—
127±6·4 (12)	90±2·9 (10)	75±2·4 (8)	—
113±4·9 (12)	—	75±4·1 (10)	—
	Females		
*110±4·4 (16)	—	66±2·9 (18)	—

* Corresponding batches.

minations were made in six of these in order to show that the activity had fallen to the level reached in the previous series (the results appear on the left of Fig. 6*b*). The remaining animals were divided into three groups, and testosterone 100 μg./day, cortisone 100 μg./day and testosterone 100 μg./day plus cortisone 100 μg./day injected into each group respectively. Fig. 6*b* shows that the mixed substances restored the normal catalase level, each singly being only partially successful. Although adrenalectomy produced a bigger drop than castration, cortisone gave a smaller rise than testosterone. Fig. 6*a* also shows the effect of giving testosterone, 100 μg./day, to animals adrenalectomized only. There is a rise which falls short of the normal level; the extent of this rise is almost identical with that resulting from the injection of testosterone into a castrated and adrenalectomized animal.

Although there is a considerable variation from batch to batch in the normal male catalase level, Table 4 shows that over a number of batches the levels after castration or adrenalectomy are remarkably constant. The mean levels in adrenalectomized females, and castrated and adrenalectomized males, are also practically identical.

DISCUSSION

Marked and rapid falls in liver catalase level have been shown to follow the operative removal of the testes in males, and the adrenals in both sexes. Post-operative anorexia appears to make no significant contribution to the observed changes. Administration of testosterone or cortisone respectively results in an almost equally rapid rise to normal. It seems clear that the sex difference in normal mice is due to lack of testicular secretion in the females.

It is not claimed, however, that the described effects specifically involve liver catalase. Sex differences in enzyme levels are not uncommon, and, for example, a decrease in rat-liver arginase activity following adrenalectomy has been reported (Folley & Greenbaum, 1947; Kochakian & Vail, 1947). However, the probability that other liver components may follow a similar pattern is not of great significance from the point of view of the present work.

Cortisone will not restore the catalase level of a castrated male to normal, and testosterone has no greater effect on an adrenalectomized than on a normal female. Further, a mixture of cortisone and testosterone is required to restore the normal activity in a castrated and adrenalectomized male, neither alone being adequate. This seems to indicate that each hormone exerts its own effect on the liver. Nevertheless, as Fig. 6 and Table 4 show, the total system in males is more complicated. Adrenalectomy produces a greater fall in male catalase level than castration. Adrenalectomy of a castrated animal results in a further significant depression, but castration of an adrenalectomized animal does not. In view of the abnormal metabolism of an adrenalectomized animal and the complexity of the known interrelations between the various hormone systems, the evidence is not sufficiently clear cut to show whether the secretion of testosterone is under adrenal control. However, as far as the system under observation is concerned, it does appear that in an adrenalectomized male, either the testes are not producing the normal quantity of testosterone, or the testosterone which is produced is incapable of exerting its normal effect. The further observation that 100 μg./day of testosterone raises the level in an adrenalectomized animal to the same point whether the testes are present or not, does suggest that adrenalectomy interferes in some way with testicular secretion.

Neither progesterone nor deoxycorticosterone will replace cortisone in restoring the catalase level of an adrenalectomized mouse, although high doses of the latter appear to have a slight effect.

The level of activity observed in castrated or adrenalectomized males is relatively constant, whereas average levels in different batches of normal mice fluctuate more widely. This suggests that the fluctuations are due to differences in the hormonal stimuli.

No work has yet been done on the possible influence of the pituitary gland on the system, but this point will be investigated.

SUMMARY

1. Castration of young adult male albino mice produces a depression in liver catalase activity. The level is restored by the injection of testosterone. Injection of testosterone into female mice elevates their lower normal level to that of the male.

2. Adrenalectomy in both sexes results in a depression in liver catalase activity. Cortisone, but not progesterone or deoxycorticosterone, restores the normal level.

3. Cortisone has little effect on the liver catalase level of castrated or normal mice, and testosterone has no more effect on adrenalectomized than on normal female mice.

4. Although adrenalectomy of castrated males results in a further fall in liver catalase level, castration of adrenalectomized males does not. The ultimate level is the same in whichever order the operations are done.

5. A mixture of cortisone and testosterone is needed to restore to normal the liver catalase activity of a castrated and adrenalectomized male, neither alone being adequate. Testosterone has the same effect on an adrenalectomized as on a castrated and adrenalectomized male.

I am grateful to Prof. S. P. Bedson, F.R.S., for his interest, and to Dr M. H. Salaman for his suggestions and advice. I am also indebted to Dr G. H. Bourne and Dr C. J. O. R. Morris for helpful discussions, to Mr L. J. Hale, Miss B. L. DeBoise and Mr K. Goodall for skilled technical assistance, and to Mr G. Downes for his care of the animals. The expenses of this research were partly defrayed out of a block grant from the British Empire Cancer Campaign.

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Exogenous and Endogenous Cytochrome *c*

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Keilin (1930) first suggested that extracted cytochrome *c* might be different from the cytochrome *c* existing in the living cell. Later, Keilin & Hartree (1940) showed that exogenous cytochrome *c* is much less active catalytically than the endogenous form. This has been re-emphasized by Keilin & Hartree (1945, 1949) and by Slater (1949*b*, 1950*b*). Keilin & Hartree (1940) also pointed out that their heart-muscle preparation contained a cytochrome system in a very high degree of organization comparable to that existing in the living cell, and this was later demonstrated quantitatively by Slater (1949*b*). In the present investigation, the quantitative difference in catalytic activities between exogenous and endogenous cytochrome *c* is further examined, a qualitative difference between the two forms is demonstrated and it is also shown that by suitable means the soluble exogenous form of cytochrome *c* can be transformed into a bound form which is identical with the endogenous form in every respect. Some of the findings have been briefly reported elsewhere (Tsou, 1951*a*).

MATERIALS AND METHODS

Cytochrome c of iron contents of 0·34 and 0·43% was prepared by the methods of Keilin & Hartree (1945). Unless otherwise specified, the preparation with 0·34% iron was used.

Dihydrocozymase. Cozymase was obtained by an unpublished method of Ochoa and reduced enzymically as described by Slater (1950*a*).

Light absorption in the visible and ultraviolet regions was measured with a Beckman photoelectric spectrophotometer.

Heart-muscle preparation containing the complete cytochrome system and the enzymes responsible for the reduction of cytochrome *c* by succinate and dihydrocozymase was prepared according to the method of Keilin & Hartree (1947) as previously described (Tsou, 1951*a*).

Extraction of cytochrome c *from washed heart-muscle mince.* Samples (6 g.) of heart-muscle mince, well washed with water until haemoglobin-free, were weighed into a series of test tubes to which were added 6 ml. portions of the extracting solutions of known concentrations. The water content of the same washed muscle mince was determined by drying a separate 6 g. sample to constant weight at 100°. The mixtures were allowed to stand at room temperature overnight, filtered, and the cytochrome *c* content of the filtrates was determined as follows: to 3 ml. of the filtrate was added 0·1 ml. of diluted (1:10) heart-muscle preparation in 0·25 M-phosphate buffer, pH 7·3, thoroughly mixed, and the optical density (D_o) at 550 mμ. read in a 1 cm. cell against a blank containing 3 ml. of 0·1 M-phosphate buffer, pH 7·3, and 0·1 ml. of the same diluted heart-muscle preparation; 0·1 ml. of a solution containing 0·4 M-succinate and 0·05 M-cyanide was then added to both the cytochrome *c*-containing solution and the blank. After mixing, the optical density was again read until a steady value (D_r) was reached. The concentration of cytochrome *c* in the extract was given by the formula

$$\frac{D_r(3{\cdot}2/3{\cdot}0) - D_o(3{\cdot}1/3{\cdot}0)}{1{\cdot}92} \times 10^{-4}\,\text{M},$$

in which the difference ($D_r - D_o$) is corrected for dilution by the added reagents and divided by the difference between the

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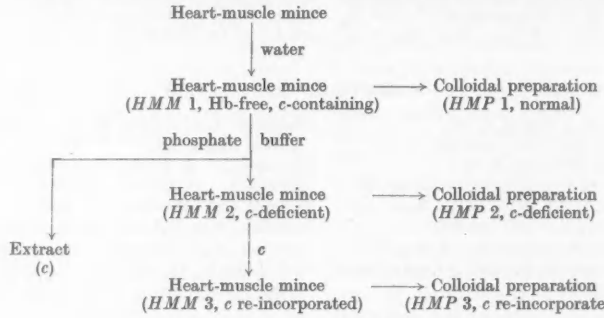
Table 1. *Relation between various colloidal heart-muscle preparations*



Hb = haemoglobin, *c* = cytochrome *c*.

molecular extinction coefficients of reduced and oxidized cytochrome *c* ($1{\cdot}92 \times 10^4$).

Cytochrome c*-deficient heart-muscle preparation* was obtained as follows: heart-muscle mince, 150 g., well washed with water, was stirred with 2 l. 0·15 M-phosphate buffer, pH 7·3, for 2–3 hr. The mince was collected on muslin, squeezed hard to remove the phosphate buffer which contained extracted cytochrome *c*, and the process repeated three times. During the last extraction, the stirring was carried out at 0° overnight. Next morning the mince was collected and squeezed as before, and washed once with water, and the cytochrome *c*-deficient muscle mince was then treated in the same way as for a normal heart-muscle preparation.

Incorporation of exogenous cytochrome c *into the cytochrome* c*-deficient muscle mince.* Heart-muscle mince (50 g.) was thoroughly washed with water and made deficient in cytochrome *c* by repeated extraction with phosphate buffer as described above. After the last extraction, the muscle mince, washed once again with water, was incubated 3 hr. at room temperature with 100 ml. of a solution containing 0·01 M-phosphate buffer, pH 7·3 and 3×10^{-5} M-cytochrome *c*. The mixture was then ground with sand and treated as for a normal heart-muscle preparation except that the precipitate obtained at pH 5·5 was washed once with water before the final suspension in phosphate buffer. The relation between various colloidal heart-muscle preparations is summarized in Table 1.

RESULTS

The cytochrome c*-cyanide complex.* The light absorption of the ferricytochrome *c*-cyanide complex in the visible region of the spectrum was determined by Potter (1941) and by Horecker & Kornberg (1946). The results obtained by these workers have been confirmed in the present study, and measurements have extended to the ultraviolet region. Incubation with 0·01 M-cyanide for 20 min. at room temperature shifts the Soret band of ferricytochrome *c* at 408 mμ. by more than 5 mμ. towards the red (Fig. 1). Cytochrome *c* preparations of both 0·34 and 0·43 % iron were used and identical results obtained.

Potter (1941) found that unlike cytochrome *c* itself, the cyanide complex is not reduced either by succinate or by dihydrocozymase in presence of the appropriate enzyme systems. It has now been found that the complex is not perceptibly reduced by *p*-phenylenediamine, ascorbic acid, cysteine, quinol or adrenaline, all of which readily reduce the untreated pigment.

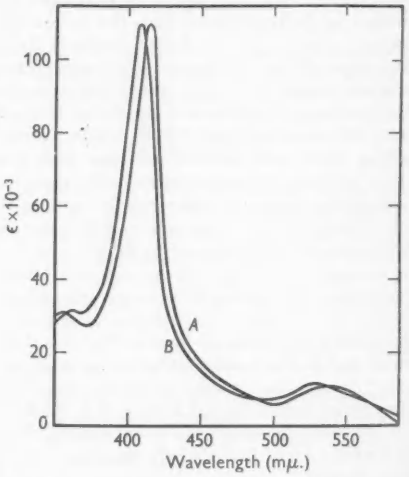


Fig. 1. The absorption spectrum of the ferricytochrome *c*-cyanide complex (*A*) compared with that of ferricytochrome *c* (*B*). Cyanide concentration, 0·05 M; readings taken after 20 min. incubation at room temperature.

The ferricytochrome *c*-cyanide complex is stable throughout the pH range 2·5–14 with the same absorption spectrum persisting. At very low pH, no change in the absorption spectrum of ferricytochrome *c* can be observed despite prolonged incubation with strong cyanide.

Behaviour of endogenous cytochrome c *towards cyanide.* Although the catalytic activities of endogenous and exogenous cytochrome *c* are of very different order, no qualitative difference between them has been reported. It has now been shown that whereas exogenous cytochrome *c* combines with cyanide to form a stable complex which is not reduced by the succinic dehydrogenase system, by the dihydrocozymase-diaphorase system, or by simple organic reducing agents such as *p*-phenylenediamine or ascorbic acid, endogenous cytochrome *c* is not affected by prolonged incubation with cyanide. The absorption spectrum of endogenous ferricytochrome *c* as present in the heart-muscle preparation cannot easily be seen. Consequently, any small changes in its absorption spectrum on incubation with cyanide cannot be detected spectroscopically. The lack of effect of prolonged incubation with cyanide on endogenous cytochrome *c* was demonstrated by the experiments set out in Table 2. It must be pointed out that: (1) In all cases when cytochrome *c* was in a partly oxidized and partly reduced state (tube no. 5) both exogenous and endogenous cytochrome *c* were present. Moreover, the intensity of the α-band of reduced cytochrome *c* corresponded exactly with the intensity of that of the endogenous cytochrome *c* had there been no exogenous cytochrome present, whereas the intensity of the band at 537 mμ. corresponded to that of the cytochrome-cyanide complex had there been no endogenous cytochrome present. Therefore, it was concluded that cyanide had combined exclusively with the exogenous form. (2) The addition of three drops of heart-muscle preparation to a total volume of 2 ml. makes no difference to the absorption spectrum as viewed in the microspectroscope. A mixture of buffer (2 ml.) with three drops of heart-muscle preparation plus a little solid sodium dithionite showed no absorption bands whatsoever when examined in the microspectroscope. (3) The absence of reduction of endogenous cytochrome *c* by succinate in tubes 5 and 7 was not due to the combination of cyanide with endogenous cytochrome *c*, but to the inactivation of succinic dehydrogenase by cyanide (Tsou, 1951*b*). (4) The reduction of endogenous cytochrome *c* by succinate plus three drops of untreated heart-muscle preparation was rather slow. This was expected because succinic dehydrogenase is destroyed on long incubation with cyanide (Tsou, 1951*b*), and the amount of active succinic dehydrogenase in three drops of heart-muscle preparation was much less than was originally present. In any case, the succinic dehydrogenase added was situated on one set of particles which, naturally, would react with the endogenous cytochrome *c* situated on another set of particles with some difficulty (see Keilin & Harpley, 1941).

From these results, it is clear that incubation with cyanide does not interfere with the reduction of endogenous cytochrome *c* by chemical or enzymic reactions. Under the same conditions, as shown above, the reduction of exogenous cytochrome *c* would be blocked completely within 20 min. It will also be noticed that the presence of denatured protein does not protect exogenous cytochrome *c* from the action of cyanide. Moreover, when both

Table 2. *Effect of incubation with cyanide on exogenous and endogenous cytochrome* c

(The filling of each tube is listed in the first part of the table. The final concentration of cyanide in all cases was 0·02 M which would suffice to convert practically all the exogenous cytochrome *c* into the complex within 20 min. Immediately before the experiments the cytochrome *c* used was acidified to pH 3, aerated to ensure complete oxidation and reneutralized. The tubes were allowed to stand at room temperature for 24 hr. Portions (2 ml.) were then removed from each tube, treated as described in the second part of the table and examined in the microspectroscope. The results are denoted by +, − and ± signs showing that the cytochrome *c* was in a reduced state, oxidized state and a partly oxidized and partly reduced state respectively.)

Filling of the tubes (ml.)	Tube no. 1	2	3	4	5	6	7
HMP 1 in 0·1 M-phosphate buffer, pH 7·4	−	−	−	8	8	8	8
Cyt. *c*, 0·5 mM, in 0·5% NaCl	2	2	2	2	2	−	−
KCN, 0·12 M	−	2	2	−	2	−	2
NaCl, 0·5%	−	−	−	−	−	2	2
KCl, 0·12 M	2	−	−	2	−	2	−
0·1 M-Phosphate buffer, pH 7·4	8	8	8	−	−	−	−
Denatured globin, mg.	−	−	50	−	−	−	−
Treated with	**Observed reduction**						
Three drops Co I H_2	−	−	−	+	±	+	+
Three drops Co I H_2 and 3 drops of *HMP* 1	+	−	−	+	±	+	+
Three drops 0·4 M-succinate	−	−	−	+	−	+	−
Three drops 0·4 M-succinate and 3 drops *HMP* 1	+	−	−	+	±	+	+
Small crystal *p*-phenylenediamine	+	−	−	+	±	+	+
Three drops 0·5 M-sodium ascorbate	+	−	−	+	±	+	+

HMP 1 = heart-muscle preparation, 1 (Table 1) undiluted. Cyt. *c* = cytochrome *c*. Co I H_2 = dihydrocozymase.

exogenous and endogenous cytochrome *c* are present, cyanide acts exclusively on the exogenous form.

As the activity of the heart-muscle preparation is rapidly destroyed at low and high pH (Keilin & Hartree, 1940), it has not been possible to determine within what pH range endogenous cytochrome *c* is resistant to the attack of cyanide. However, experiments carried out as above at pH 6·2 and 8·4 yielded identical results, hence it is concluded that endogenous cytochrome *c* does not combine with cyanide within the pH range where the colloidal succinic system is active.

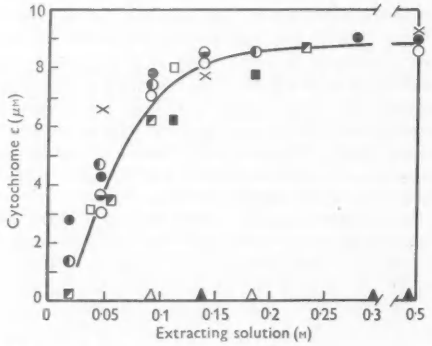


Fig. 2. Extraction of cytochrome *c* from heart-muscle mince (for method see text). ●, KCl; ○, NaCl; ◐, LiCl; ◒, KNO_3; phosphate buffer: ■, pH 6·2; ◪, 7·3; □, 8; ×, Na_2SO_4; △, boric acid titrated to pH 7·3; ▲, sucrose.

Extraction of cytochrome c *from water-washed heart-muscle mince.* In spite of very thorough washing of the heart-muscle mince with water, very little, if any, cytochrome *c* is removed, although it is well known that exogenous cytochrome *c* is extremely soluble in water. However, the bound cytochrome *c* which is present in the heart-muscle mince could be extracted by salt solutions as is shown in Fig. 2. (The bound cytochrome *c* in the final colloidal preparation was, however, not extracted by similar treatment, nor was it extracted by trichloroacetic acid in the pH range 1·5–5.) All the salt solutions tested had the same order of effectiveness except borate buffer which was completely non-effective. Non-electrolytes, such as sucrose, were without effect. It appears that the extent of extraction is mainly dependent on the anion concentration of the extracting solutions. Cytochrome *c* thus extracted has the same activity in biological systems as the pigment prepared in the usual way and, like the latter, it is also susceptible to the attack of cyanide.

Cytochrome c*-deficient heart-muscle preparation and the difference in activity between endogenous and exogenous cytochrome* c

The study of the effect of added cytochrome *c* on respiration has been somewhat handicapped by the fact that the most active cell-free preparation

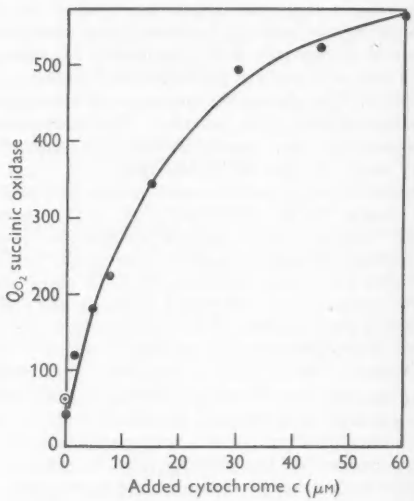


Fig. 3. Effect of added cytochrome *c* on the succinic oxidase activity of a cytochrome *c*-deficient heart-muscle preparation. Succinic oxidase activity measured at 38° as described by Slater (1949*b*). The point (⊙) shows the effect of added denatured globin. (Q_{O_2} = μl. O_2 uptake/mg. fat-free dry wt. of heart-muscle preparation/hr.)

(Keilin & Hartree, 1940), which behaves almost exactly like the natural complex oxidation system of the cell, already contains enough cytochrome *c* for its normal function. For instance, under optimal conditions, the addition to such preparations of an excess of cytochrome *c* only raises the rate of oxidation of succinate by about 40% (Slater, 1949*b*). Although exogenous cytochrome *c* is freely soluble in water, the particle-bound endogenous pigment of the heart-muscle preparation survived thorough washing with water and remained firmly attached to the particles. So far it has not been possible to remove the bound cytochrome *c* from the heart-muscle preparation without harming the latter.

By extraction with phosphate buffer, it has now been found possible to remove the greater part of the bound cytochrome *c* from heart-muscle mince (*HMM* 1, Table 1) without affecting the ability of the final preparation (*HMP* 2, Table 1) to oxidize succinate when cytochrome *c*, extracted and purified in the usual way, is added. On reduction with

succinate, such a preparation (*HMP* 2) shows strong *a* and *b* bands but a very weak *c* band. The cytochrome *c* content, estimated as described by Slater (1949*b*), is about 0·1 μmol./g. fat-free dry weight of heart-muscle preparation as compared with 0·76 of a normal preparation. The Q_{O_2} (μl. O_2 uptake/mg. fat-free dry wt./hr.) of this preparation (*HMP* 2) in the oxidation of succinate with different concentrations of added cytochrome *c* is given in Fig. 3.

It will be seen that the cytochrome *c*-deficient preparation, in presence of excess added cytochrome *c*, has a $Q_{O_2} = 565$, which is of the same order as that of a normal preparation. Although the addition of denatured globin has little effect on the activity of the cytochrome *c*-deficient preparation, the addition of 6×10^{-5} M-cytochrome *c* to this preparation caused a more than 10-fold increase as compared with 1·3- to 1·4-fold in a normal preparation which in absence of any added cytochrome *c*, already has a Q_{O_2} about 450. The addition of about $2{\cdot}7 \times 10^{-5}$ M-cytochrome *c* is necessary in order to raise the Q_{O_2} of this preparation (*HMP* 2, Table 1) to about 450 which is the activity under the same experimental conditions of a normal preparation containing about $2{\cdot}3 \times 10^{-7}$ M-endogenous cytochrome *c*. These results are similar to those obtained by Keilin & Hartree (1949) while using certain modified types of preparation and support their conclusion that cytochrome *c* acts as a specific oxido-reduction catalyst and not as an indifferent protein in the succinic oxidase system.

The cytochrome oxidase activity of the cytochrome *c*-deficient preparation was about double that of a normal heart-muscle preparation (Table 3). This is probably due to the removal of a considerable amount of indifferent protein by phosphate buffer extraction. However, the succinic dehydrogenase activities of these two preparations were about the same (Table 3), which indicates that besides indifferent protein, part of the dehydrogenase had also been removed or destroyed.

Table 3. *Comparison of a normal and a cytochrome c-deficient heart-muscle preparation*

(The succinic oxidase and succinic dehydrogenase activities were measured as described by Slater (1949*b*). The cytochrome oxidase activities were measured by both the Keilin & Hartree method (1940) and the Slater method (1949*a*). Q_{O_2} = μl. O_2 uptake/mg. dry wt. of enzyme preparation.)

	Heart-muscle preparations	
	Normal	Cytochrome *c*-deficient
Succinic dehydrogenase (Q_{O_2})	250	290
Cytochrome oxidase (Q_{O_2}):		
Keilin & Hartree	1470*	2800*
Slater	3300	6550
Succinic oxidase (Q_{O_2})	590	565

* These values are based on total dry weight, whereas the others are based on fat-free dry weight of the enzyme preparations.

Transformation of exogenous cytochrome c *into the bound form*

By incubation of the cytochrome *c*-deficient heart-muscle mince (*HMM* 2, Table 1) with cytochrome *c* as described above, a final colloidal preparation (*HMP* 3, Table 1) was obtained into which cytochrome *c* was re-incorporated (Table 4). This cytochrome *c* was firmly bound to the colloidal particles and could not be removed by thorough washing with water, it therefore behaved like endo-

Table 4. *Relation of cytochrome* c *content and succinic oxidase activity of different heart-muscle preparations*

(Three different preparations* were used: a normal preparation (normal), a cytochrome *c*-deficient preparation (*c*-def.) and a preparation with re-incorporated cytochrome *c* (*c*-re-incorp.). The determination of cytochrome *c* content and the measurement of succinic oxidase activity were carried out as described by Slater (1949*b*). The amount of enzyme preparation used per flask and the concentration of added cytochrome *c* in each case are listed in the table.)

		Heart-muscle preparations		
		Normal	*c*-def.	*c*-re-incorp.
Fat-free dry wt./ml. (mg.)		19·2	22·6	17·4
Cytochrome *c* content (μmol./g. fat-free dry wt. of preparation)		0·76	0·092	0·62
Amount of preparation/flask (mg.)		0·96	1·13	0·87
Concentration of endogenous cytochrome *c* under experimental conditions (μM)		0·22	0·031	0·164
	Added cytochrome *c* (μM)			
Succinic oxidase (Q_{O_2})	0	455	62	355
	5	520	200	470
	15	—	370	—
	60	620	555	605

* All these three preparations were made from the same batch of heart-muscle mince (see Table 1).

genous cytochrome *c* in a normal heart-muscle preparation. Moreover, like the normal, but unlike the cytochrome *c*-deficient preparation, this preparation with re-incorporated cytochrome *c* (*HMP* 3, Table 1) already contained enough cytochrome *c* for the near optimal functioning of the whole succinic oxidase system. Thus the addition of excess cytochrome *c* raised the succinic oxidase activity from a Q_{O_2} of 355 to 605, as is shown in Table 4.

The turnover number, i.e. the oxygen uptake/min./oxygen equivalent of cytochrome *c* (Keilin & Hartree, 1940), of bound cytochrome *c* can be calculated from the data given in Table 4. It was obtained by dividing the succinic oxidase activity without added cytochrome *c* (in μl. O_2 uptake/min./mg. fat free dry weight) of the preparation by its bound cytochrome *c* content (in oxygen equivalent/mg. fat-free dry weight of the total preparation). The values were 1780 and 1710 for the bound cytochrome *c* present in the normal preparation (*HMP* 1) and in the preparation with re-incorporated cytochrome *c* (*HMP* 3, Table 1) respectively. The turnover number of exogenous cytochrome *c*, on the other hand, was 24 (obtained from Table 4 by dividing the increase in oxygen uptake of the cytochrome *c*-deficient preparation by the oxygen equivalent of cytochrome *c* added). Similar results were obtained with dihydrocozymase as substrate. The activity of the re-incorporated cytochrome *c* is thus of the same order as that of endogenous cytochrome *c* but far greater than that of exogenous cytochrome *c*. Experiments similar to those of Table 2 also showed that the re-incorporated cytochrome *c*, like endogenous cytochrome *c*, does not combine with cyanide.

DISCUSSION

In the preparation with re-incorporated cytochrome *c* (*HMP* 3, Table 1), and in the normal preparation (*HMP* 1, Table 1) the activity of bound cytochrome *c* is lower than the figure given by Keilin & Hartree (1940) for the cytochrome *c* of yeast cells, i.e. turnover number, 3850. This is understandable, since it can hardly be expected that the heart-muscle preparation has retained completely the high state of organization and efficiency of the living cell (Slater, 1949*b*), or that cytochrome *c* present in the heart-muscle preparation is entirely in the endogenous form. In support of this view, the reducible fraction of cytochrome *c* in the heart-muscle preparations, after prolonged incubation with cyanide, is always somewhat less than the total cytochrome *c* present.

Quantitative differences between exogenous and endogenous cytochrome *c* have been previously pointed out. Keilin & Hartree (1940) showed that even under optimal conditions, the exogenous pigment is only about one-third as active as the endogenous form. Keilin & Hartree's results on the exogenous pigment were obtained by adjusting the concentrations of heart-muscle preparation and of added cytochrome *c* so as to make the latter the limiting factor. On the other hand, the activity of the endogenous pigment was measured under such conditions that there was no reason to suppose that cytochrome *c* was the limiting factor. In the present work, it is demonstrated that using approximately the same amount of heart-muscle preparations (Table 4) the difference in activities of the two forms of cytochrome *c* is far greater. Slater (1950*b*) has shown that exogenous cytochrome *c* is reduced by succinate or dihydrocozymase in presence of the appropriate enzyme systems at least 1200 times more slowly than the endogenous pigment. In addition, exogenous cytochrome *c* is reduced by cysteine (Keilin, 1930) or ascorbic acid (Slater, 1949*a*) more readily than the endogenous enzyme. However, their different behaviour towards incubation with cyanide seems to be the first record of any qualitative difference. In view of the fact that cytochrome *c* occupies an important position in the electron transferring chain of biological oxidation, and since it is the only component of the cytochrome system so far prepared in a soluble and pure form, a considerable amount of work has been done to link various oxidative enzymes to the cytochrome system via exogenous cytochrome *c*. It would appear, therefore, that the demonstration of any qualitative difference between the two forms of the pigment might assist towards a clearer understanding of the part played by cytochrome *c* in biological oxidation. The present work emphasizes the necessity for caution in interpreting results obtained with exogenous cytochrome *c*, and for confirmation of the results, whenever possible, with an enzyme material, such as the heart-muscle preparation of Keilin & Hartree, where the cytochromes are in a more organized state. Experiments with a cytochrome *c*-deficient heart-muscle preparation indicate that it is much more difficult to 'saturate' the oxidase with exogenous cytochrome *c* than with the endogenous form (Fig. 3). In other words, the presence of 0·22 μM-endogenous cytochrome *c* in the manometer flask is already enough for the near optimal function of the succinic oxidase system of a normal preparation, whereas a far greater concentration of exogenous cytochrome *c* was necessary before the curve in Fig. 3 (where a cytochrome *c*-deficient preparation was employed) approached an asymptotic value.

There are two possible explanations for the differences between exogenous and endogenous cytochrome *c*. (1) During extraction, cytochrome *c* was slightly modified so that the soluble 'pure' cytochrome *c* obtained was chemically different from the endogenous pigment present in the living

cell. (2) The difference in activity between the two forms of cytochrome *c* was purely a matter of proper orientation of the cytochrome *c* molecule in relation to other components of the cytochrome system (Keilin & Hartree, 1940). Exogenous cytochrome *c* may be less active because, unlike the endogenous form, it is not firmly linked to the colloidal particles and therefore not so readily accessible to the other components of the succinic oxidase system.

The observation that cyanide, although a small molecule, can react only with the exogenous form favours the first explanation, so does the observation that non-enzymic reducers such as ascorbic acid and cysteine reduce the two forms of cytochrome *c* at different speeds. These facts are difficult to understand if the second explanation is accepted. The finding that under suitable conditions the transformation of the endogenous to the exogenous form of cytochrome *c* can be reversed favours the second explanation. It also seems to be unlikely that the very mild treatment of phosphate buffer extraction can have modified the endogenous molecule to any appreciable extent.

However, these two views are not actually mutually incompatible. Keilin & Hartree (1940, 1949) and Slater (1949*c*) have shown that the colloidal structure of the enzyme-carrying particles in the heart-muscle preparation is of paramount importance. As far as cytochrome *c* is concerned, its proper orientation within a highly specific spatial arrangement of the respiratory pigments is essential for its optimal activity. In other words, endogenous cytochrome *c* is so situated on the colloidal carrier particles that it is readily available to its electron donors and acceptors. It can also be assumed that this orientation is realized through a linkage which binds cytochrome *c* to its proper place in the colloidal carrier particles of the heart-muscle preparation. By disruption of this linkage with salt solutions or trichloroacetic acid treatment, not only is cytochrome *c* released from the colloidal particles and the very specific spatial arrangement of the respiratory pigments disturbed, but also the resulting pigment, exogenous cytochrome *c*, is then made susceptible to the attack of cyanide.

SUMMARY

1. The absorption spectrum of the ferricytochrome *c*-cyanide complex, given by Horecker & Kornberg for the visible region, has been extended to the ultraviolet region and was found to be substantially different from that of ferricytochrome *c* itself. The complex is stable in all but very acid solutions (pH 2·5).

2. Spectroscopic observations showed that the cytochrome *c* as present in the Keilin & Hartree heart-muscle preparation (endogenous cytochrome *c*) does not combine with cyanide at physiological pH.

3. Cytochrome *c*, as present in the heart-muscle mince, can be transformed into the soluble exogenous form by extraction with salt solutions. By removing cytochrome *c* from a water-washed heart-muscle mince with phosphate buffer a cytochrome *c*-deficient heart-muscle preparation was obtained. Experiments with this preparation showed, in agreement with earlier results, that exogenous cytochrome *c* is catalytically much less active than the endogenous form.

4. The transformation of endogenous cytochrome *c* into the exogenous form is reversible; when the exogenous pigment is again incorporated into the colloidal particles of the heart-muscle preparation by suitable means, it then assumes the characteristic properties of the endogenous form.

5. The relation of the spatial arrangement of the respiratory pigment to the activity of cytochrome *c* has been discussed. It is suggested that endogenous cytochrome *c* is bound to the colloidal carrier particles through a linkage which cannot be disrupted without causing profound changes in the catalytic and other properties of endogenous cytochrome *c*.

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The Combustion of Tritium-Labelled Organic Compounds

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The tritium (T) in solids can be determined directly only on small amounts of material, and then only with poor efficiency, owing to absorption by the sample of the low energy tritium β-rays. The tritium in water, however, can be estimated with high efficiency by reducing the water to hydrogen, and measuring the currents obtained through hydrogen-filled ion chambers (Henriques & Margnetti, 1946; Kamen, 1947); or, more conveniently, by preparing tritio-methane from the water and determining the tritium content in a proportional counter (White, Campbell & Payne, 1950). Thus the estimation of the tritium content of organic substances will usually necessitate their combustion to water; a technique suitable for the routine combustion of organic compounds for this purpose has been devised.

METHOD AND APPARATUS

Kamen (1947) has recommended the combustion techniques developed for deuterium assays as suitable for the combustion of tritiated metabolites, but states that contamination must be constantly guarded against by testing for radioactivity in water obtained from the combustion of samples of inactive material. If activity is found in the blanks the combustion tube has to be flushed several times by burning inactive hydrogen compounds until satisfactory blanks are obtained. Such a procedure will be too tedious for routine analysis.

Possible causes of contamination of the apparatus are retention of water in porous materials, and exchange of tritium with labile hydrogen of substances such as glass. The copper oxide filling and the quartz or glass tubes used in most combustion methods are porous, the porosity of the quartz increasing markedly after several analyses. Tritium is known to cling tenaciously to glass (Williams & Ronzio, 1950) and Geiger counters are difficult to decontaminate after they have contained THO (Pace, Kline, Schachman & Harfenist, 1947). By using deuterium, Rittenberg (1948) has found that a baked and evacuated Pyrex vessel appears to have a layer of water 3 molecules deep over its entire surface, although the method did not differentiate between absorbed water and OH groups of silicates. For these reasons it seemed advantageous to use an all-metal combustion apparatus.

The metal tube must have high resistance to oxidation at high temperatures, since part of the combustion tube is exposed to O_2 at 1000°. Kamen (1947) states that monel metal reaction vessels used for reduction of THO are easier to decontaminate than vessels made from stainless steel, suggesting that high nickel content alloys would be desirable. For these reasons inconel tubes were tried. These were obtained from Henry Wiggin and Co. Ltd., London. Some scale is formed inside these tubes after repeated use, but their life is very long and in this respect they are much superior to quartz tubes.

The copper oxide filling used in the deuterium techniques has been replaced by the Pt catalyst introduced by Kopfer (1878). Kopfer used platinum black. Dennstedt (1897) modified the method to use strips of platinum foil. The authors use a roll of 150 mesh platinum gauze 20 cm. long. In order to ensure complete oxidation and to limit poisoning of the catalyst the platinum is kept at 1000°.

The apparatus is shown in Fig. 1.

O_2 from a rubber bladder of 20 l. capacity is sucked through the apparatus by a small mechanical pump. A metal reservoir of 5 l. capacity between the pump and the rest of the apparatus prevents transient variations in O_2 flow. The rate of flow is controlled by a valve connecting the reservoir to air. This arrangement results in a very steady flow of O_2 through the apparatus without the necessity of refilling Mariotte bottles, and has been found to prevent the uneven burning of the sample which sometimes occurs in Pregl combustion trains.

Water and volatile impurities are removed from the O_2 by the spiral copper trap *A*, immersed in dry ice-propanol. The sample is inserted through the removable gas-tight breech. The boat containing the sample is mounted in the holder *D*, attached to the block *C*, which fits loosely in the combustion tube. The rapid flow of O_2 between *C* and the combustion tube limits back diffusion of volatile materials.

A combustion of 500–600 mg. of substance proceeds smoothly to completion if the sample-heating furnace is raised at a constant rate from room temperature to 700° in 30–45 min., and is maintained at this temperature for 15 min. Even volatile substances burn steadily from a silica boat

in these conditions. The time between successive analyses has been reduced to a few minutes by using an unlagged sample heating furnace which rapidly combustion tube. An electric clock driving a 'Simmerstat' control automatically raises the temperature of the sample furnace.

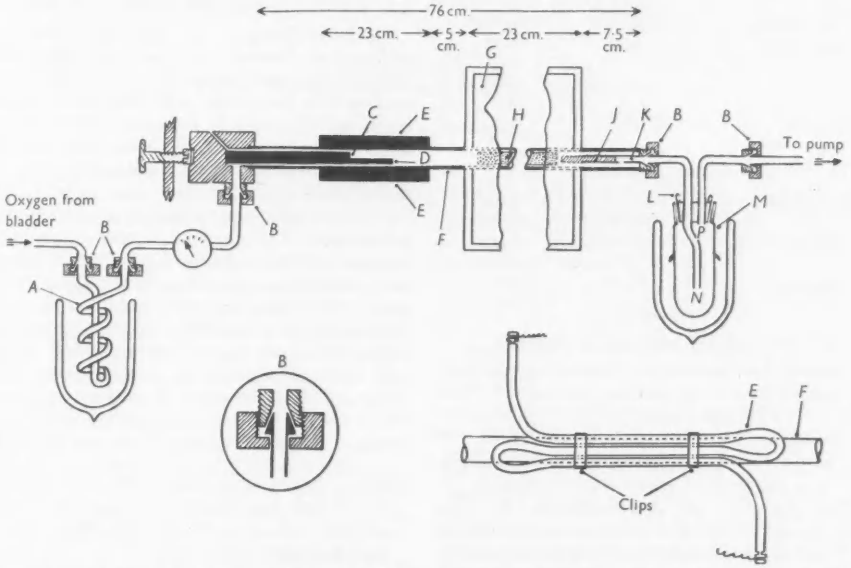

Fig. 1. Apparatus for combustion of tritium-labelled organic compounds. *A*, spiral made from $\frac{3}{8}$ in. outside diam., $\frac{1}{4}$ in. inside diam. copper tube, 6 turns, 1 turn/in.; *B*, brass crush nipples; *C*, see text; *D*, boat holder 7 cm. long; *E*, sample heater; *F*, inconel tube $\frac{3}{8}$ in. inside diam., $\frac{5}{8}$ in. outside diam.; *G*, main furnace at 1000°; *H*, roll of platinum gauze, 20 cm. long; *J*, copper rod; *K*, copper tube; *L*, brass B29 cone joint; *M*, Pyrex vessel, 3·2 cm. inside diam., 15 cm. overall length; *N* and *P*, copper tubes for attachment of anhydrone trap.

cools. In the present apparatus the sample is heated by two unlagged heaters *E*, each consisting of a 700 W. electrical element supported by

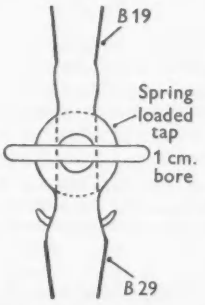

Fig. 2. Adapter for connecting Pyrex vessel to apparatus for tritium estimation.

magnesia packing in an inconel sheath which was obtained from Hotpoint Ltd., London. Each heater is folded as shown in Fig. 1, and is clamped along the

The water from the combustion is collected in the Pyrex vessel *M* immersed in dry ice-propanol, and attached to the combustion tube by the cone joint *L*. *L* is lubricated with silicone high vacuum grease. The copper rod *J* and tube *K* conduct sufficient heat from the furnace zone to prevent condensation within *K*. It has been found that at the O_2 flow rates normally used (20–50 ml./minute) 90–95 % of the water of combustion is collected in *M*. For specific activity measurements quantitative collection is unnecessary. For accurate determination of the efficiency of combustion a $CaCl_2$ and an anhydrone trap are connected between *P* and *N*.

After attachment to *M* of the adapter shown in Fig. 2, the specific activity of the water is determined by the method already described (White *et al.* 1950).

RESULTS

Completeness of combustion

Evidence for the completeness of combustion is contained in Table 1.

Table 1. *Percentage hydrogen content of test substances measured with the combustion apparatus*

Substance	Hydrogen content (%) Calculated	Observed
Benzoic acid	4·92	4·96
		5·00
		4·92
		4·94
Resorcinol	5·46	5·53
		5·50
Vanillin	5·26	5·06
		5·24
β-Naphthol	5·56	5·45
		5·56
Dextrose	6·67	6·63
		6·59
Cholesterol	11·9	12·1
		11·8

Preparation of tritiated test substance

A tritiated test substance of unknown composition was prepared by heating 0·3 ml. of THO (activity $1{\cdot}4 \times 10^8$ counts/sec.) with 2 g. β-naphthol in an evacuated Pyrex ampoule in the presence of active nickel for 16 hr. at 175°. The resulting substance was dissolved in ether and crystallized. It was then vigorously stirred with 200 ml. of boiling water, allowed to cool and then thoroughly shaken with 50 ml. of ether in a separating funnel. The ether phase was separated and the ether evaporated at room temperature. The crystals were again treated with 30 ml. of boiling water and the substance extracted with ether. The radioactivity in the water used for the second washing was only 19·2 counts/sec./ml., showing that most of the labile or semi-labile tritium attached to or in the vicinity of oxygen atoms had been removed. After evaporation of the ether the specific activity of the substance was found to be 21·9 counts/sec./mg.

Test for contamination of apparatus

To test the contamination of the apparatus 123 mg. of the test substance (activity $2{\cdot}93 \times 10^3$ counts/sec.) were burned. The apparatus was then flushed with oxygen at 30 ml./min. for 15 min., followed by combustion of a sample of inactive dextrose. The water from the dextrose had an activity of only 1·2 counts/sec. corresponding to a contamination of only 0·04%. In biological experiments samples will rarely contain more than 200–300 counts/sec., so that the above contamination is negligible.

Substances containing nitrogen and sulphur

Oxides of nitrogen and sulphur, formed by the combustion of substances containing these elements, will combine with some of the hydrogen. It was necessary to prove that this combination does not result in differences in the specific activity of the water collected. The tritiated test substance (63·3 mg.) was dissolved in ether and 10 g. of β-naphthol added. The ether was evaporated. The ratio of test substance to β-naphthol is such that the percentage of hydrogen in the mixture can be taken as equal to that of β-naphthol. Glycine (343·5 mg.) and 1-amino-2-naphthol-4-sulphonic acid (122·3 mg.) were added to 857·7 mg. of the mixture, and the whole was carefully mixed by grinding in a small mortar. From the figures in Table 2 it can be seen that the presence of sulphur and nitrogen is without effect on the specific activity of the water obtained. It is thus unnecessary to introduce reagents for the retention of nitrogen and sulphur.

Table 2. *Specific activities of water from combustion of tritiated test substance + β-naphthol, with and without dilution by glycine and 1-amino-2-naphthol-4-sulphonic acid*

Substance burned	Specific activity of water (counts/sec./g.) Calculated	Observed
Test substance + β-naphthol	—	276
	—	276
	—	269
Test substance + β-naphthol + glycine + 1-amino-2-naphthol-4-sulphonic acid	131 131	136 128

SUMMARY

1. An apparatus has been developed for the routine combustion of tritiated metabolites. Combustion is complete, rapid and smooth.

2. The apparatus shows negligible contamination and the analyses are not influenced by the presence of nitrogen or sulphur.

The authors would like to thank Prof. B. S. Platt for his interest in this work, and Mr D. Rivers for constructing the apparatus and for valuable suggestions as to its design.

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The Fermentation of Pentoses and Uronic Acids by Bacteria from the Rumen Contents of Sheep

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Although the fermentation of cellulose in the rumen of sheep and cattle has received considerable attention (Vaartiovaara, Suomalainen & Arhimo, 1944; Elsden, 1945; Marston, 1948; Sijpesteijn, 1948; Hungate, 1950), comparatively little work has so far been carried out on the almost equally important hemicellulose fermentations which occur when grass, straw and hay is fed to the animal (cf. McAnally, 1942). This is most certainly owing to the difficulties of obtaining sufficient quantities of a purified substrate which has not been degraded during chemical treatment.

The hemicelluloses occurring in greatest quantity in natural fodders are those composed mainly of xylose and uronic acids, with arabinose and galactose occurring to a lesser extent (Norman, 1937; Weihe & Phillips, 1940; Whistler, 1951). It has been demonstrated that in the ruminant, approximately 50% of the pentosans of the feed are utilized (McCollum & Brannon, 1909; Fraps, 1930) and that approximately 30–40% is utilized in the rumen (Marshall, 1949), and since pentosans may account for 20–25% of the dry matter of the feed (Fraps, 1930) the fermentative breakdown of these substances assumes considerable importance. Further, since pentose sugars are probably not readily utilized by the animal body, they will be of greater value to the ruminant if they are converted to other products by microbial fermentation.

As a preliminary to a study of pentosan fermentation it was considered that the study of the fermentation of the readily available xylose would be of value and isolations of xylose-fermenting organisms from the rumen of sheep were attempted, under conditions believed to be not too widely different from those in the rumen.

The liquid in the rumen consists of sheep saliva diluted to some extent with plant juices and water consumed. The main form of nitrogen is ammonia, but small quantities of organic nitrogen are also present. In addition rumen liquor contains growth factors synthesized by the micro-organisms. It is neutralized by the acidic products of fermentation, chiefly volatile fatty acids and carbon dioxide. The composition of sheep saliva as determined by McDougall (1948) shows it to be an alkaline bicarbonate and phosphate buffer. This formed the basis for the salt medium used by Elsden (1946) to study '*in vitro*' fermentations of rumen micro-organisms. A similar medium has been employed in this study, suitably modified to enable pure culture isolations to be carried out.

METHODS

Experimental animals and withdrawal of rumen liquor. The sheep used were two 1-year old Cheviot wethers fitted with ruminal and duodenal cannulae (Phillipson & Innes, 1939; Phillipson, 1948), and were maintained on a meadow-hay diet. The hay, of which a large stock had been set aside, was fed to the sheep twice daily at 12 hr. intervals and the food and water were removed 2 hr. after feeding. This practice prevents any changes occurring in the rumen during the next 10 hr. owing to a fresh influx of food and water. Samples were withdrawn through the rumen cannula by means of a wide-bore glass tube, 2 hr. after feeding, and were strained through six layers of surgical gauze before use.

Preparation of media

Liquid salt basal medium reproducing rumen conditions. The basal acetate/salt medium had the following composition: 0·15M-KCl 36 ml., 0·11M-$CaCl_2$ 8 ml., 0·15M-KH_2PO_4 8 ml., 0·15M-$MgSO_4$ 8 ml., 0·15M-$(NH_4)_2HPO_4$ 40 ml., acetic acid (glacial) 6·3 ml., water to 900 ml. Sterilized at 120° for 20 min. Sodium carbonate (12·6 g.) was sterilized dry, in a separate container.

The sterile carbonate, when added to the sterile salt medium, produced a medium at pH 6·8 buffered with bicarbonate and acetate and saturated with CO_2. The supplement of rumen liquor was then added.

The basal phosphate salt medium was prepared as follows. Double strength basal salt medium (450 ml.) in which the acetic acid had been replaced by 1 ml. of syrupy phosphoric acid was sterilized at 120° for 20 min. 450 ml. of 3% (w/v) $NaHCO_3$ was sterilized by Seitz filtration under a slight vacuum and added to the salt medium. If necessary, a final adjustment of the pH to 6·8–7·0 was made with sterile syrupy phosphoric acid. Cleared and sterilized rumen liquor supplement (100 ml.) was then added.

Sterilization of supplement containing growth factors and organic nitrogen. The gauze-strained rumen samples were centrifuged at 2500 *g* for 1 hr. and the pH of the supernatants was adjusted to 6·8 with glacial acetic acid. The liquid was centrifuged at 25,000 *g* for 15 min. and the almost clear supernatants were passed through a Seitz filter and stored at 5°. Under these conditions there was no change in the pH owing to loss of CO_2 during manipulation.

The supplement added to the phosphate salt medium did not contain additional acetic acid and was prepared as rapidly as possible to prevent alkalinity developing.

The calculated ionic composition of the isolation medium is shown in Table 1.

Table 1. *Composition of the basal medium used for the isolation of xylose-fermenting bacteria*

Ion	Concentration (calc.) (mg./100 ml.)	Concentration in sheep saliva (McDougall, 1948) (mg./100 ml.)
Na	493	370–462
K	39	16–46
Ca	5	1·6–3·0
Mg	6	0·6–1·0
NH_4	20 (as N)	9–20 (as N)
SO_4	10·5	—
Cl	20	25–43
P	36	37–72
HCO_3	1000	440
AcO	100	—

Sterile rumen liquor 10–15% (v/v).

Preparation of solid medium. This was prepared by mixing, at 50°, equal volumes of 4% (w/v) neutral agar-agar (Baird and Tatlock Ltd.) containing 0·05M-K_2HPO_4, and the complete basal medium containing the carbohydrate supplement, and then pouring into Petri dishes and cooling rapidly in the refrigerator. Under these conditions, no loss of CO_2 occurs sufficient to cause the plates to become alkaline within 20 min. As an additional check on the process, a few drops of bromothymol blue indicator were incorporated into some of each batch of plates. If the indicator became blue the batch was discarded.

Fermentations

Mixed rumen micro-organisms. The apparatus consisted of Büchner flasks, fitted with rubber stoppers containing an inlet capillary and carrying Bunsen valves on the side arm (cf. Elsden, 1946). These were warmed to 40° before use.

The medium, also at 40°, was Elsden's (1946) buffer without modification, containing 14 g. $NaHCO_3$/l. and saturated with CO_2 prior to use. Under these conditions the initial pH was 7·1–7·3.

Inoculum. A sample of rumen contents was collected, strained through gauze as described above, and added to the medium to bring the concentration to 20% (v/v). The sugars were added to the medium prior to inoculation. After the addition of the inoculum the contents of the flasks were gassed with CO_2. This procedure was carried out whenever a sample was removed from the flasks during fermentation. Incubations were for 18–20 hr. at 40°, the flasks being shaken every half-hour for the first 8–10 hr. The fermentations became very vigorous in 2–4 hr. Since the initial concentration of $NaHCO_3$ was high, the pH did not fall below 6·6–6·8 during the fermentation.

Analytical methods

Carbohydrates were estimated in the fermentation solutions by the method of Hagedorn & Jensen (1923) after removal of the protein by $ZnSO_4$ and NaOH (Neish, 1946). Standard calibration curves were prepared for each carbohydrate.

Volatile acid. Total volatile acids were determined by distillation of samples in the Markham still (Markham, 1942). The fermentation liquid (10 ml.) was pipetted into the still followed by 8 g. of $MgSO_4.7H_2O$, 5 ml. of 10% (w/v) sodium tungstate and two drops of *n*-octanol; 4 ml. of 10N-H_2SO_4 were rapidly added and the stopper was inserted in the still. Each 50 ml. of distillate was titrated in a stream of CO_2-free air with carbonate-free 0·05N-NaOH until the titration reached the blank value, phenol red being the indicator.

This procedure enabled the presence of lactic acid to be detected, since all the volatile acids higher than acetic distilled in the first three fractions. Formic acid was completely distilled in five fractions. If lactic acid was present, no more than seven fractions were collected. In these instances the titrations were 0·1–0·2 ml. above the blank.

The bulked fractions were distilled with HgO and $MgSO_4$ (Friedemann, 1938) to remove formic and lactic acid. The distillates were titrated with NaOH and evaporated to dryness, the final evaporation being carried out in a micro-Kjeldahl flask, and the acids were extracted from the sodium salts as described by Elsden (1946).

The individual acids in the extract were fractionated and estimated on buffered silica columns (Moyle, Baldwin & Scarisbrick, 1948). By this procedure, acetic, propionic and *n*-butyric acids in a mixture were distilled and fractionated with the average recoveries shown in Table 2.

Table 2. *Recovery of fatty acids after distillation and analysis by chromatography* (*Moyle* et al. 1948)

Acid (and source)	Recoveries (%) Mean	Range
Acetic acid (A.R. Na salt)	99·5	87–113
Propionic acid (from recrystallized propionamide)	89·7	82–94
Butyric acid (B.D.H. reagent Na salt)	92·3	89–95

Formic acid. Estimated by distillation of a fresh sample of fermentation liquid and bromine oxidation of the concentrated distillate (Neish, 1946).

Lactic acid. Determined by the method of Friedemann & Graeser (1933) in the fermentation liquid cleared of protein by $Zn(OH)_2$ and treated with copper/lime (Van Slyke, 1917) to remove residual carbohydrates and acetoin.

Ethanol. The volatile neutral fraction calculated as ethanol was determined in two ways. On a large sample, 10 ml. of the neutral, cleared, solution and 10 g. of NaCl were steam distilled in the Markham still and the first 50 ml. containing the ethanol were collected. Samples were estimated for volatile neutral substances by dichromate oxidation (see Neish, 1946). On a small scale the method of Winnick (1942) was used.

Succinic acid. By the method of Krebs (1937), using sheep heart succinoxidase.

2:3-Butanediol and acetoin. These were not differentiated, but were estimated as total periodate-oxidizable substances by the method of Neish (1946) and calculated as butanediol.

Sugars

D-(+)-Xylose and D-(+)-cellobiose were bacteriological sugars obtained from Thos. Kerfoot. L-(+)-Arabinose was from L. Light and Co., D-(+)-galacturonic acid from Roche Products Ltd. D-Glucuronolactone (supplied by the courtesy of Corn Products Ltd., New York), was given by Dr G. A. Levvy. The lactone was converted to the sodium salt by electrometric titration to pH 8·0 with caustic soda. D-(+)-Glucose was British Drug Houses 'Analar' anhydrous dextrose.

The isolation of xylose-fermenting organisms

Inoculations from *in vitro* xylose fermentations, carried on for 24 hr. only, were made into a sterile basal medium containing 0·4% xylose (w/v) contained in sterile 50 ml. conical flasks fitted with rubber stoppers and a Bunsen valve. The flasks were incubated for 18–24 hr. at 40°. Streak plates were made from the second or third enrichment cultures and were incubated under an atmosphere of 95% N_2 and 5% CO_2 at 40°.

Colonies were picked into xylose basal medium, and the subsequent growth was again streaked on agar. An isolate was not considered pure unless two such passages through liquid and solid medium yielded identical homogeneous colonies which appeared to consist of morphologically similar organisms. The cultures were stored in stabs in basal+agar medium at 2°. No organism was considered worth studying unless four loops of a 24 hr. culture inoculated in 100 ml. of basal medium completely fermented 0·4% xylose in 12–18 hr.

RESULTS

Fermentations with mixed rumen micro-organisms

The volatile fatty acids formed by the fermentation of xylose and glucuronic acid are presented in Tables 3 and 5, which show that acetic acid predominated. Lactic acid was absent in both fermentations. Microscopic examination of the fermentation liquids showed that a large number of small rod-like organisms were present, and from these xylose enrichment cultures the organisms described below were isolated.

Very little volatile acid was produced in the absence of added substrate. The increases were from 0·4 to 0·6 mmol. total volatile acids per 100 ml. fermentation liquid in 20 hr., and of this acetic acid accounted for approximately 50% and propionic and butyric acids for almost equal quantities of the remainder. The quantities were too small to be accurately estimated by the methods used.

Pure cultures

Seventeen strains were isolated and five of these were studied in more detail. These five were Gram-negative rods with slightly rounded ends, 1 μ. wide and 3–5 μ. long. On agar in 24 hr., circular colonies 1·5–2·00 mm. wide were formed which showed a

Table 3 (*a*). *The products of fermentation of xylose by mixed micro-organisms from the rumen of sheep fed on hay*

(The carbohydrate was completely fermented in each case. Values in mmol. and in mg. atoms carbon.)

Exp. no.	1		2		3		4		5	
Product	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon	mmol.	mg. atoms carbon
Acetic acid*	1·63	3·26	1·41	2·82	2·62	5·24	2·5	5·0	1·90	3·80
Propionic acid*	0·85	2·55	1·24	3·72	0·42	1·26	0·50	1·50	0·51	1·52
Butyric acid†	0·31	1·24	0·30	1·20	0·71	2·84	0·65	2·60	0·57	2·28
Formic acid	0·39	0·39	0·34	0·34	0·80	0·80	0·10	0·10	0·10	0·10
Lactic acid	0	0	0	0	—	—	—	—	—	—
Total carbon	—	7·44	—	8·08	—	10·14	—	9·20	—	7·70
Total volatile fatty acids	2·78	7·05	2·95	7·74	3·75	9·34	3·15	9·10	2·98	7·60
Xylose added	4·01	20·05	4·27	21·35	3·62	18·10	3·62	18·10	3·62	18·10

* All values are corrected for the original content of the acids in the added rumen liquid.

† Butyric acid, as estimated by the method of Moyle *et al.* (1948), may contain *iso*butyric acid.

Table 3 (*b*). *The percentage of the carbon of xylose fermented by mixed rumen micro-organisms found in the acetic and propionic acids produced, and the molar ratios of the products formed to each other and to the substrate fermented*

(Calculated from the data in Table 3(*a*))

Exp.	Total carbon recovered (%)	Ratios (mmol.) Volatile acid / Substrate used	Ratios (mmol.) Acetic / Propionic	Carbon recovered As acetic acid (%)	Carbon recovered As propionic acid (%)
1	36·2	0·70	1·63	16·0	12·4
2	37·8	0·69	1·12	13·2	17·4
3	55·8	1·0	6·5	29·0	7·0
4	50·8	0·87	5·0	27·6	8·3
5	42·5	0·71	3·8	21·0	8·4

Table 4. *Fermentation reactions given by cultures isolated from xylose-enrichment cultures of mixed rumen micro-organisms*

(A.G., Acid + Gas; 0, no reaction; –, not tested; V.P., Voges-Proskauer reaction. All cultures grew on peptone-NaCl without further additions.)

Culture	V.P. reaction	Gelatin stab	Citrate	Xylose	Glucose	Lactose	Glycerol	Sorbose	Mannose	Raffinose	Maltose	Sucrose	Dulcitol	Mannitol
1	+	0	0	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	A.G.	A.G.	A.G.	A.G.
14	+	0	0	A.G.	A.G.	A.G.	A.	0	A.G.	A.	A.	0	A.	A.G.
15	+	0	0	A.G.	A.G.	A.G.	A.	0	A.G.	A.	A.	0	A.G.	A.G.
16	+	0	0	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	A.G.	A.	A.	A.G.
17	+	0	–	A.G.	A.G.	A.G.	A.G.	0	A.G.	A.G.	–	–	–	–

greenish opalescence in transmitted light. The organisms were non-motile, facultative anaerobes.

In Table 4 are shown the distinctive fermentation reactions of these organisms, together with some characterizing reactions. The organisms appear to correspond to intermediate strains of *Escherichia coli*, but differ mainly in the ability to produce small quantities of acetoin and to ferment cellobiose. The non-utilization of citrate indicates that the organisms are of the intestinal type, rather than soil or water organisms (Topley & Wilson, 1946).

Fermentations with pure cultures of the organisms

The pure strains were inoculated into the phosphate basal medium containing the carbohydrates known to be components of hemicelluloses, and after incubation for 24 hr. the cultures were analysed for volatile acids, ethanol, and lactic acid and periodate-oxidizable substances. The fermentation liquids of culture 14 were also analysed for succinic acid. The results are presented in Table 6.

DISCUSSION

It has been found possible to prepare, in a relatively simple way, both liquid and solid media which resemble the rumen liquid of animals fed on hay. The importance of obtaining such conditions has been stressed by a few workers. Hungate (1942) showed that the osmotic pressure of synthetic media must be identical with that of the rumen liquid in order to culture certain rumen protozoa. Vaartiovaara *et al.* (1944) stressed the importance of bicarbonate buffers in order to reproduce the conditions necessary for the rapid fermentation of cellulose by rumen enrichment cultures, while Elsden (1946), Sijpesteijn (1948) and Hungate (1950) also used bicarbonate-buffered medium in their *in vitro* studies. Only Elsden (1946), however, appears to have paid attention to the high salt concentrations also necessary.

The sterilization of the rumen-liquid supplement which has been added to such media has received less attention. Hungate (1950) autoclaved rumen liquid under a carbon dioxide atmosphere, while Sijpesteijn (1948), recognizing that the liquid became alkaline on heating, removed excess bicarbonate by means of calcium chloride, before sterilization by heat. Since the composition of rumen liquid is unknown as regards growth factors, it seems more desirable to sterilize by Seitz filtration and to adjust the pH with an acid naturally occurring in the rumen, in order to prevent alkalinity developing.

Such media are low in organic nitrogen, allowing the carbohydrate source to be the major selective factor in *in vitro* studies, thus decreasing the possible isolation of artifacts and of fermentation products

Table 5. *The products of the fermentation of glucuronolactone by mixed rumen micro-organisms*

(The carbohydrate was completely fermented in each case.)

Product	Exp. 1 mmol.	Exp. 1 mg. atoms carbon	Exp. 1 Total carbon (%)	Exp. 2 mmol.	Exp. 2 mg. atoms carbon	Exp. 2 Total carbon (%)
Acetic acid	2·36	4·72	37·4	1·70	3·40	27·0
Propionic acid	0·23	0·69	5·4	0·36	1·08	8·6
Butyric acid	0·19	0·76	6·0	0·08	0·32	2·5
Total carbon recovered	—	6·17	—	—	4·80	—
Glucurone used	2·1	12·6	—	2·1	12·6	—
% Carbon recovered	—	—	48·8	—	—	38·1
Ratio: Acetic / Propionic	10·0	6·9	—	4·8	3·1	—

Table 6. *Products of fermentation of carbohydrates by five strains of coliform organisms isolated from enrichment cultures of rumen micro-organisms*

(No propionic acid was produced in any fermentation. The carbohydrates were completely fermented in all cases. Values in mmols.)

Product	Xylose	Glucuronic acid	Glucose	Cellobiose	Xylose	Glucuronic acid	Glucose	Cellobiose
	Strain 1				Strain 14			
Formic acid	0·84	0·69	1·36	3·08	0·87	1·31	1·36	1·66
Acetic acid	0·71	1·13	0·92	0·94	0·88	1·09	1·40	0·90
Lactic acid	0	0	0·95	0·23	0	0·06	0·55	0·23
Succinic acid	0	—	—	—	0	0	0	0
Ethanol	0	0	0·56	0	—	0	0·51	0
Acetoin and butane-2:3-diol	0	0	0·54	0·16	0	0	0·20	0·15
Substrate fermented	1·75	1·1	2·5	1·68*	1·75	1·1	2·5	1·68*
	Strain 15				Strain 16			
Formic acid	1·00	0·66	1·85	N.U.	0·57	0·75	1·85	1·44
Acetic acid	0·71	1·03	1·37	N.U.	0·82	0·75	0·90	1·14
Lactic acid	0	0	0·48	N.U.	0	0	0·83	0·17
Succinic acid	0	—	—	N.U.	0	—	—	—
Ethanol	0·20	0	0·62	N.U.	0·35	0	0·61	0
Acetoin and butane-2:3-diol	0	0	0·20	N.U.	0	0	—	0
Substrate fermented	1·75	1·1	2·5	N.U.	1·75	1·1	2·5	1·68*
	Strain 17							
Formic acid	0·71	0·94	1·83	1·34				
Acetic acid	1·09	0·91	0·77	0·94				
Lactic acid	0	0·08	0·95	0·23				
Succinic acid	0	—	—	—				
Ethanol	0·44	0	0·08	0				
Acetoin and butane-2:3-diol	0	0	0·26	0·16				
Substrate fermented	1·75	1·1	1·75	1·68*				

* Calculated as glucose. N.U., not utilized.

derived from the organic nitrogen source (cf. Dubos & Miller, 1937). Under such conditions it may be tentatively assumed that the organisms isolated carry out degradations by metabolic pathways not entirely dissimilar to those that they would utilize during growth within the rumen. This assumption cannot yet be tested and is made with considerable reserve.

The products of fermentation of xylose by mixed rumen micro-organisms (Table 3) clearly indicate the predominance of acetic acid which accounts for an average of 21·3 % of the total carbon fermented, while propionic acid accounts for an average of 10·7 %. The most striking feature of Table 6 is the marked difference in the products of fermentation of xylose and glucuronic acid on the one hand, and glucose and cellobiose on the other. Lactic acid, acetoin and 2:3-butanediol are not formed from xylose and glucuronic acid, but are formed from glucose and cellobiose. No volatile alcohol is produced from cellobiose or glucuronic acid, while only three strains produced volatile alcohol from xylose.

These results are distinctly different from those of Stanier & Adams (1944), Adams & Stanier (1945),

Breden & Fulmer (1931), and Reynolds & Werkman (1937), in which the fermentation of hexoses and pentoses by members of the Enterobacteriaceae yielded substantially the same products from both types of substrate. The present view of the metabolism of the hexoses by coliform organisms involves at one stage the formation of pyruvic acid as an intermediate (Stephenson, 1949). The distinction between the fermentation end products of the xylose and glucuronic acid on the one hand, and glucose and cellobiose on the other, would therefore suggest that, in the case of the first two carbohydrates, either pyruvic acid is not formed or else the mechanisms involved in the further formations of both lactic acid and acetyl methyl carbinol are not operative; for example, there may be some necessary intermediate product involved which is formed only during the fermentation of glucose and cellobiose. It is clear, however, that the fermentation of the hexuronic acid is not comparable with that of the homologous hexose, and the relationship, if any, between the metabolism of glucuronic acid and xylose is being investigated.

SUMMARY

1. A procedure is described for the preparation of solid and liquid media which closely reproduce conditions in liquid from the rumen of sheep.

2. Using these media seventeen strains of Gram-negative coliform rods of an intermediate type have been isolated in pure culture.

3. The products of fermentation (formic acid, acetic acid, lactic acid, ethanol, butyleneglycol and acetoin) formed by five of these strains from xylose, glucuronic acid, glucose and cellobiose, show marked differences both as regards the substrate and the strains of organism used.

4. The fermentation of xylose and glucuronic acid appears to follow a pathway different from that followed in the fermentation of glucose.

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Biochemistry of the Wood-rotting Fungi

7. METABOLIC PRODUCTS OF *POLYPORUS BENZOINUS* (WAHL.) FR.

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The fungus *Polyporus benzoinus* (Wahl.) Fr., although comparatively rare in Britain, is fairly common on the Continent, particularly in Sweden, where it attacks coniferous logs, especially *Abies* species. It produces in culture a marked aromatic odour and for this reason came under examination in our investigation of the odoriferous products formed by some of the wood-rotting fungi (Birkinshaw & Findlay, 1940; Birkinshaw, Bracken & Findlay, 1944; Birkinshaw & Morgan, 1950). The present communication gives a complete account of this work so far as it has proceeded. A summary of our findings has already appeared (Morgan, 1951).

In preliminary investigations the fungus was grown on aqueous synthetic medium, containing glucose, nutrient salts and Marmite. Ether extraction of the wet minced mycelium gave a crude mixture of metabolic products with a pronounced aromatic odour. Treatment of the ether residue with fresh ether gave a white powder, acid to litmus, of m.p. 285–290° (decomp.). The ether filtrate after removal of this sparingly soluble acid was extracted with aqueous sodium bicarbonate. Acidification of the separated bicarbonate layer afforded a white precipitate of a complex aliphatic acid, of m.p. 53–54·5°. The bicarbonate-extracted ether layer, after removal of solvent, left a fatty residue which still retained the aromatic odour. Steam distillation of this material gave a colourless aromatic oil which was identified as anisaldehyde. This substance is probably the main contributor to the odour of the fungus in culture. Anisaldehyde has already been reported as one of the metabolic products of *Trametes suaveolens* (Birkinshaw *et al.* 1944).

The odorous constituent having been identified, further attention was given to the solid substances extracted from the wet mycelium by ether. The medium so far employed gave poor growth and mycelial development, and towards the end of the long incubation period there were signs of incipient autolysis. After continued cultivation of the fungus on this medium the yields of the two acidic products fell almost to zero. A search for a more suitable medium led to the use of an aqueous Vimaltol-Marmite medium which permitted vigorous growth and gave much enhanced yields of the acidic products, in addition to the odorous constituent.

The extraction procedure with the new medium was similar to that previously adopted, but it was found that when the ether filtrate was treated with aqueous sodium bicarbonate the separated bicarbonate layers gave on standing a crystalline sodium salt of the aliphatic acid. The salt was only slightly soluble in cold water and was obtained in almost pure condition, much purer than the acid which had previously been obtained by acidification of the total bicarbonate layer.

In the later stages of the work the mycelium, on removal from the culture flasks, was submitted to the action of a high-speed disintegrator. This reduced it to a viscous paste and considerably shortened the time necessary for its complete extraction by ether. In addition, it led to a further increase in the amount of the aliphatic acid recovered as the sodium salt. The acid of m.p. 285–290° (decomp.) was found by analysis to have a composition corresponding with $C_{30}H_{46}O_4$, or possibly $C_{30}H_{48}O_4$. It gave characteristic colour reactions with the Liebermann reagent and with the Salkowski reagent. Ethereal diazomethane converted it into a neutral monomethyl ester $C_{31}H_{48}O_4$ (or $C_{31}H_{50}O_4$), m.p. 192–193°. Acetylation, preferably at room temperature, gave a crystalline diacetate, m.p. 212–214° (decomp.), titrating in the cold as a monobasic acid. When subjected to ozonolysis in chloroform solution it afforded 53% of 1 mol. of formaldehyde.

These properties indicate that the substance is in all probability a dihydroxymonocarboxylic acid of formula $C_{30}H_{46}O_4$, and possessing a terminal methylene group. It would seem to fall into the group of triterpenes, a number of which occur naturally and have already been described. Amongst these the triterpene alcohols, basseol, lupeol ($C_{30}H_{50}O$; Heilbron, Moffat & Spring, 1934) and betulin ($C_{30}H_{50}O_2$; Vesterberg, 1923) are each characterized by the presence of an exocyclic methylene group. A comparison of the acid from *Polyporus benzoinus* with certain triterpenoid acids isolated from *P. betulinus* by Cross, Eliot, Heilbron & Jones (1940) shows striking points of resemblance. These authors minced the fresh fungus, as found in nature, and extracted it with cold ethanol and subsequently under reflux with a mixture of acetone and

ether. From the ethanolic extract after saponification they isolated a mixture of sterols and a crystalline acid $C_{30}H_{48}O_4$ or $C_{36}H_{50}O_4$, m.p. 194°, which they named polyporenic acid *A*. Hydrolysis of the acetone-ether extract yielded two further acids, polyporenic acid *B*, m.p. 300–310° (decomp.), of similar composition to *A*, and, in small amount, polyporenic acid *C* m.p. 270–275°. The last named acid was analysed only as its methyl ester, $C_{31}H_{48(50)}O_4$, m.p. 192–193°. The melting point of the free polyporenic acid *C* and of its methyl ester and the analysis of the methyl ester were so close to those of our triterpenoid acid that the possibility arose that the two acids might be identical. This possibility was further strengthened by the observation that the colour reactions of our acid with the Liebermann and Salkowski reagents were similar to those described for polyporenic acid *C*. Through the courtesy of Prof. E. R. H. Jones, F.R.S., and Dr T. G. Halsall of Manchester University, a small sample of the methyl ester of polyporenic acid *C* was made available to us. The sample showed no depression in melting point when mixed with our methyl ester, indicating that our acid was in fact identical with polyporenic acid *C*.

The suggestion of Cross *et al.* (1940) that polyporenic acid *C* may be identical with gypsogenin would appear to be discounted if the identity of polyporenic acid *C* with our triterpenoid acid is accepted, since ozonolysis of the latter has afforded evidence of a terminal methylene group which is not present in gypsogenin. Further, gypsogenin contains an aldehyde group, which cannot be present in our triterpenoid acid since all the oxygen atoms are otherwise accounted for, two being present in a carboxyl group and one in each of two hydroxyl groups.

Cross *et al.* (1940) put forward an interesting speculation as to the possible interrelationship between the acids of the parasitic fungus, *P. betulinus*, and the dihydric triterpene alcohol betulin, $C_{30}H_{50}O_2$, which forms a major constituent of the bark of the host tree (*Betula alba*). This hypothetical relationship involves the interchange of a methyl and carboxyl group ($CO_2H \rightleftarrows CH_3$), and would apparently imply that the triterpenoid acids found in the fungus are derived with some modification from the host tree and are not elaborated by the parasite. The present work shows that a fungal species of the same genus, *Polyporus benzoinus*, is capable of synthesizing one of these triterpenoid acids when grown on a synthetic medium containing only glucose (and a little Marmite) as source of carbon. Hence it appears likely that *P. betulinus* has the same power of synthesis of the triterpenoid nucleus and is not dependent on the host plant for its supply of this complex molecule.

It is interesting to note that the polyporenic acids *A*, *B* and *C* were isolated after saponification of the extracts of the naturally occurring sporophore of *P. betulinus*. Extracts from mycelium of *P. benzoinus* cultured by us in the laboratory yielded the acid *C* directly without previous saponification, indicating that, in this fungus, it occurs in the free state.

Further examples of triterpenoid acids derived from wood-rotting fungi have recently been described. Eburicoic acid, $C_{30}H_{48}O_3$, m.p. 293°, was first isolated by Kariyone & Kuruno (1940) from *Fomes officinalis* Fr. and was further characterized by Gascoigne, Holker, Ralph & Robertson (1950, 1951) and by Lahey & Strasser (1951) who obtained the acid from *Polyporus anthracophilus* Cooke. It was also isolated from the mycelium of *P. eucalyptorum* Fr., *P. sulphureus* (Bull.) Fr. and *Lentinus dactyloides* Cleland. The acid contains in addition to carboxyl a secondary hydroxyl and an exocyclic methylene group.

From *Trametes odorata* (Wulf.) Fr., Gruber & Proske (1950) isolated trametenolic acid, $C_{30}H_{48}O_3$, m.p. 259–261°, which also contains a secondary hydroxyl group.

The crystalline sodium salt obtained from the bicarbonate extraction of the ether extract of the mycelium is converted on acidification into one or other of two compounds depending on the procedure employed. The more stable form, m.p. 53–54·5°, is prepared by acidification of an aqueous solution of the sodium salt. Analysis indicates the formula $C_{22}H_{36}O_5$ or $C_{22}H_{38}O_5$. The equivalent determined by titration in the cold is intermediate between the values required for a dibasic and a tribasic acid; after refluxing with excess of standard alkali the acid becomes fully tribasic. Acidification of the hydrolysate leads to recovery of the original acid of m.p. 53–54·5°. If the argument which follows as to the function of the five oxygen atoms is accepted, namely that three are present in an acid anhydride grouping and two in a lactone group, then the formula $C_{22}H_{38}O_5$ is untenable, since the formula $C_{22}H_{36}O_5$ would represent a fully saturated compound with no carbocyclic ring.

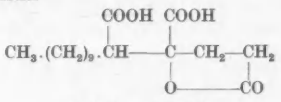
The less stable form of the acid is prepared by shaking a suspension of the sodium salt in ether with dilute hydrochloric acid (2N). It has m.p. 78° and is less soluble in organic solvents than the lower melting form, particularly in light petroleum. Analysis of this acid indicates that it is a hydrated form of the acid of m.p. 53–54·5°, agreeing with a formula of $C_{22}H_{38}O_6$ with a little additional water. This acid is readily dehydrated to the acid of lower melting point; the melting point falls from 78 to 55–65° on keeping for 6 weeks at room temperature in a stoppered tube. The simplest interpretation of this behaviour is to regard this dehydration as due to the formation of an acid anhydride. We therefore

propose for the higher melting acid the name of ungulinic acid derived from *Ungulina fuliginosa* (Scop.) Pat, a synonym of *Polyporus benzoinus* (Wahl.) Fr. The acid of lower melting point then becomes ungulinic acid anhydride.

Ungulinic acid is apparently optically inactive. Ethereal diazomethane converts it into a dimethyl ester $C_{24}H_{42}O_6$, a pale-yellow oil. This ester is convertible into a trihydrazide, m.p. 190–195° (decomp.), which could not be recrystallized. Both ungulinic acid and its anhydride give colour reactions which are identical with those described for agaricic acid, which is a hexadecylcitric acid.

The neutral sodium salt of ungulinic acid which crystallizes from the bicarbonate layer during the extraction process has a sodium content in agreement with that of the disodium salt $C_{22}H_{36}O_6Na_2$. The equivalent of this salt, determined by titration after refluxing with excess standard alkali, agrees with the formula $C_{22}H_{36}O_6Na_2$ reacting as a monobasic acid. If a stream of carbon dioxide is passed through the neutralized solution after titration, a crystalline precipitate of a monosodium salt is obtained and when this is decomposed with ether-dilute hydrochloric acid, ungulinic acid is recovered.

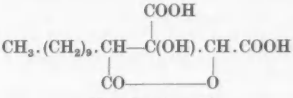
Fusion of the disodium salt with potassium hydroxide afforded a mixture of stearic, acetic and oxalic acids. A consideration of the experimental results indicates that ungulinic acid probably bears a general structural relationship to certain polybasic aliphatic acids with long carbon chains which are metabolic products of other fungi and of lichens. Amongst these are spiculisporic, minioluteic and agaricic acids.



Spiculisporic acid

(Clutterbuck, Raistrick & Rintoul, 1931; Asano & Kameda, 1941)

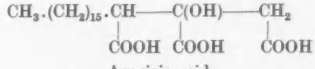
Penicillium spiculisporum Lehman



Minioluteic acid

(Birkinshaw & Raistrick, 1934)

Penicillium minio-luteum Dierckx.



Agaricic acid

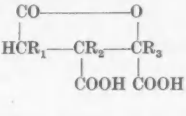
(Thoms & Vogelsang, 1907)

Fomes officinalis Fr.

Like these acids ungulinic acid is potentially tribasic. If we assume that the three acidic groups are carboxyls, then the stable form of the acid $C_{22}H_{36}O_5$ must be accounted for by anhydride formation. This indicates a constitution of a substituted succinic or glutaric acid type. Tetraethylsuccinic acid (Walker & Walker, 1905) shows a similar ready convertibility to the anhydride, since this change occurs on melting the acid or on keeping it in aqueous solution for 2 days. The third carboxyl group is assumed to be present in lactonic form since the disodium salt is neutral, yet one acid equivalent is revealed on alkaline hydrolysis. This again emphasizes the relationship with spiculisporic and minioluteic acids. Since the lactone ring appears to be stable and readily reformed after being opened by alkaline hydrolysis, ungulinic acid is probably a γ-lactone. The formation by ungulinic acid of a dimethyl ester with diazomethane, giving rise to a trihydrazide, is again evidence for the lactone structure.

The precipitation of the monosodium salt after complete hydrolysis of ungulinic acid with excess alkali and passage of carbon dioxide is in line with the observation of Hesse (1898) on roccellic acid which is an α-methyl-α'-decylsuccinic acid. Hesse found that passage of carbon dioxide through a solution of the acid in potassium carbonate gives rise to the deposition of a crystalline precipitate of the monopotassium salt.

The production of stearic acid on fusion with potassium hydroxide indicates the presence of a long aliphatic side chain and again stresses the resemblance to the other fungal and lichen acids cited. In view of these similarities it is permissible to favour a substituted tricarballylic acid configuration for ungulinic acid, which might then be represented by one of the three formulae:



I $R_1 = C_{16}H_{33}$, $R_2 = R_3 = H$.

II $R_2 = C_{16}H_{33}$, $R_1 = R_3 = H$.

III $R_3 = C_{16}H_{33}$, $R_1 = R_2 = H$.

One point which arises in connexion with this formulation calls for further comment. A natural product of the above configuration would be expected to be optically active since II and III contain two and I contains three asymmetric carbon atoms. It is, of course, possible that a racemic compound is produced as in the case of fumarylalanine, a metabolic product of the mould *Penicillium reticulosum* (Birkinshaw, Raistrick & Smith, 1942). Alternatively, the rotation may be so small as to escape observation. In the case of the mould product palitantin, the substance was reported as optically inactive (Birkinshaw & Raistrick, 1936) but an examination of some of its crystalline derivatives

and breakdown products revealed optical activity (unpublished observation). It is not possible on the evidence so far obtained to advance a definite structural formula for ungulinic acid. The substituted tricarballylic acid formulae are to be regarded as purely tentative and are advanced merely as a basis for further work on the constitution of ungulinic acid which is to be undertaken.

EXPERIMENTAL

Organism

The culture, F.P.R.L., No. 137A, obtained from the Forest Products Research Laboratory, Princes Risborough, had originally been prepared from the tissue of a sporophore of *Polyporus benzoinus* (Wahl.) Fr. found on a fallen trunk of silver fir, *Abies alba*, in Norfolk.

Preparation of the inoculum

The culture was maintained in the laboratory on malt agar slopes. The culture used for inoculation was developed on a moist Norway spruce sawdust medium containing 10% of the accelerator exactly as used in the investigation of species of *Endoconidiophora* (Birkinshaw & Morgan, 1950). The fungus grew slowly throughout the sawdust medium, and progress of the infection could be followed visibly by the spread of the white hyphae of the developing mycelium.

Media

The following media were employed:

(I) A synthetic medium containing glucose, 30 g.; KH_2PO_4, 2·5 g.; NH_4Cl, 2·5 g.; $MgSO_4$, 1·0 g.; Marmite, 1·0 g.; $FeSO_4.7H_2O$, 0·01 g.; $ZnSO_4.7H_2O$, 0·01 g.; $MnSO_4.4H_2O$, 0·005 g.; $CuSO_4.5H_2O$, 0·005 g.; $Na_2B_4O_7.10H_2O$, 0·005 g.; tap water to 1 l.

(II) An aqueous solution of Vimaltol (4% w/v) and Marmite (0·1% w/v). Vimaltol is a proprietary preparation prepared from malt extract, calcium glycerophosphate, orange juice, iron and ammonium citrate, halibut oil, vitamin D_2 and vitamin A concentrate. Marmite, also a proprietary preparation, is a concentrated extract obtained from autolysed yeast. It contains vitamins of the B complex.

Method of culture

The medium was distributed (350 ml./flask) in 1 l. conical flasks which were plugged with cotton wool and sterilized by autoclaving at 110° for 30 min. The flasks were inoculated by dropping in a little of the infected sawdust and were then incubated in the dark at 24°. In preliminary work the best time of harvesting was decided by examining single flasks at weekly intervals after the mycelium completely covered the surface of the culture fluid. The weight of ether-extractable material in the wet minced mycelium was determined in each case. After 10 weeks the weight of ether extract did not increase appreciably. The residual sugar in the culture filtrate estimated polarimetrically as glucose (medium I) had fallen to about 1·5% and the pH was approximately 4·0. The period of about 10 weeks was therefore adopted as the standard time of incubation.

Isolation of metabolic products

On the synthetic medium (I). The total wet mycelium from forty-five culture flasks was roughly minced and mechanically stirred under ether for several hours. The solvent layer was removed and the operation repeated until the extract, after removal of ether, weighed less than 0·1 g. The separate ether layers were combined, dried over Na_2SO_4 and evaporated. A viscous fawn-coloured paste (7·25 g.) remained, which was acid to litmus and possessed an aromatic odour.

This ether residue when treated with fresh ether (200 ml.) did not all redissolve. The white insoluble portion when collected and washed with ether formed a white powder (0·11 g., m.p. 285–290°, decomp.) with acid reaction to litmus.

The red-brown ether filtrate obtained after removal of the product of m.p. 285–290° was extracted with aqueous $NaHCO_3$ (2% w/v, 3 × 50 ml.). Acidification to congo red of the combined aqueous layers with dilute HCl gave a semi-solid light brown precipitate (1·96 g.). This was collected and recrystallized from light petroleum (b.p. 60–80°) at low temperature, giving ungulinic acid anhydride as a colourless crystalline product (0·33 g., m.p. 53–54° after previous slight shrinkage), reacting acid to litmus. The bicarbonate-extracted ether layer was washed with water, dried over Na_2SO_4 and evaporated to leave a yellow-brown waxy solid which still retained the aromatic odour. Steam distillation of this residue gave a colourless distillate with a colourless oil floating on the surface. This oil was collected by ether, and after removal of solvent weighed 0·61 g. The oil (0·15 g.) in ethanol (3 ml.), when treated with Brady's reagent (100 ml.), gave a characteristic 2:4-dinitrophenylhydrazone (0·30 g.). Recrystallization from ethyl acetate or xylene afforded glistening bright-red needles, m.p. 248–250°. The melting point was not depressed on admixture with the corresponding derivative of authentic anisaldehyde, m.p. 248–250°. Brady (1931) recorded a melting point of 250° for this compound. (Found: C, 53·0; H, 3·9; N, 17·3. Calc. for $C_{14}H_{12}O_5N_4$: C, 53·2; H, 3·8; N, 17·7%.)

On the aqueous Vimaltol-Marmite medium. Mycelial development on this medium was much healthier in appearance and more vigorous than when the fungus was grown on the synthetic medium. The incubation period was the same, viz. 10 weeks, and the method of isolation of the acidic metabolic products was similar, but the yields of the two acids previously encountered were greatly improved.

The triterpenoid acid of m.p. 285–290° (decomp.), was obtained in the same manner as described above. Owing to its slight solubility in ether and to the increased amount present, it began to precipitate during the early stages of evaporation of the ether extract.

After removal of the triterpenoid product by filtration, the filtrate was shaken as before with aqueous $NaHCO_3$. Owing to the increased amount of ether-soluble acid present, there was greater tendency to emulsification during the extraction and separation, particularly with the first extraction, was very slow. After combining the aqueous layers the last traces of ether were removed *in vacuo*, when the aliphatic acid began to precipitate as a sodium salt in the form of microcrystalline glistening leaflets. As it was only sparingly soluble in cold water it was well washed until free from $NaHCO_3$. When dry it formed a light, white powder with an odour of a higher fatty acid, e.g. palmitic acid.

Table 1. *Yields of products obtained from* Polyporus benzoinus

(Medium II; 10 weeks' incubation.)

No. of flasks	Weight of crude extract (g.)	pH of culture filtrate	Yield of acid (m.p. 285–290°, decomp.) (g.)	Yield of aliphatic acid as Na salt (g.)
75	13·78	3·9	4·26	2·08
96	18·08	3·9	1·65	2·37
100	19·20	3·9	3·02	2·32
100	23·83	4·2	3·39	2·26
99	20·77	4·0	4·27	2·73
99	27·18	3·8	4·14	5·19
97	22·40	3·9	2·75	4·12
98*	26·40	3·9	3·22	9·46

* Using high-speed disintegrator.

Acidification of the filtrate after removal of the sodium salt gave only a slight amount of oily material from which none of the acid of m.p. 53–54° could be obtained on treatment with light petroleum.

The culture filtrate exhibited no antibacterial activity against *Staphylococcus aureus*.

On a later batch the effect was tried of submitting the mycelium to the action of a high-speed disintegrator. This reduced it to a viscous paste and considerably shortened the time required for complete extraction with ether. It also markedly increased the recovery of the low melting-point acid (as the sodium salt).

The yields of acids obtained on medium II by the procedure outlined are given in Table 1.

Investigation of the triterpenoid acid

Properties. The acid is fairly soluble in pyridine, moderately so in ethanol in the cold. It is moderately soluble in hot glacial acetic acid, dioxan, $CHCl_3$ and nitrobenzene, but only sparingly in acetone and ethyl acetate. When crystallized from acetic acid it separates in microcrystalline aggregates.

The behaviour of the acid on heating in the melting-point tube is as follows: at 250° it begins to discolour, at 275–280° it begins to soften and the colour deepens from yellow to dark brown. It melts at 285–290° with liberation of gas. The recrystallized acid was analysed after drying in high vacuum at 100–110°. (Found: C, 76·1, 76·4; H, 10·1, 10·0. $C_{30}H_{46}O_4$ requires C, 76·7; H, 9·9; $C_{30}H_{48}O_4$ requires C, 76·3, H, 10·3%.) $[\alpha]_{5461}^{20°} + 8·19°$ (*c*, 5·1) in pyridine.

Equivalent. The acid (24·0 mg.) dissolved in ethanol (10 ml.) required 0·50 ml. of 0·1 N-NaOH for neutralization to phenolphthalein. This indicates an equivalent of 480, or if the acid is monobasic a mol.wt. of 480 ($C_{30}H_{46}O_4$ requires 471; $C_{30}H_{48}O_4$ requires 473).

Colour reactions. With $CHCl_3$ and H_2SO_4 it forms an orange lower layer. With acetic anhydride and H_2SO_4 it affords a blue-violet colour. These colour reactions are identical with those described for polyporenic acid *C* by Cross *et al.* (1940).

Methyl ester. The acid (0·50 g.) suspended in acetone (20 ml.), when treated with excess ethereal diazomethane, slowly passed into solution. After 3 hr. at room temperature the solvent was removed and the residue dried *in vacuo* over P_2O_5. Recrystallization of the product (0·49 g.; m.p. 160–170°) from methanol afforded homogeneous needles, m.p. 192–193°, after slight softening from 190°. $[\alpha]_D^{21°}$ in $CHCl_3 + 19·5°$ (*c*, 6·7); $[\alpha]_{5461}^{21°}$ in $CHCl_3 + 22·3°$ (*c*, 6·7). (Found: C, 76·9; H, 9·7; OMe, 6·3; $C_{31}H_{48}O_4$ requires C, 76·8; H, 10·0; OMe, 6·4; $C_{31}H_{50}O_4$ requires C, 76·5; H, 10·4; OMe 6·4%.) The methyl ester of polyporenic acid *C* is stated by Cross *et al.* (1940) to have m.p. 192–193°, after softening from 189°. The melting points of the methyl ester of polyporenic acid *C* obtained from Dr T. G. Halsall and of the methyl ester of the *Polyporus benzoinus* acid were compared under the same conditions of heating with the following results. Methyl ester of polyporenic acid *C*, m.p. 190–191°; methyl ester of *P. benzoinus* acid, m.p. 191–192°; mixed m.p. 191–192°.

The ester does not titrate with standard NaOH in the cold.

Diacetate

Hot acetylation. The acid (1·0 g.) was refluxed with acetic anhydride (7 ml.), acetic acid (5 ml.) and anhydrous sodium acetate (2 g.) for 4 hr. When the reaction mixture was cooled and decomposed with ice water a gummy product was precipitated. Recrystallization of this material (1·20 g.) from glacial acetic acid gave only a small yield (0·1 g.) of the diacetate, m.p. 212–214° (decomp.).

Cold acetylation. The acid (2·0 g.) was dissolved in pyridine (12 ml.) and acetic anhydride (3·0 ml.) was added. The reaction mixture was kept at room temperature overnight and then poured into iced water. The white flocculent precipitate was collected, dried and recrystallized from hot glacial acetic acid to which just sufficient water was added to induce turbidity. The product (0·5 g.), rectangular plates, had m.p. 212–214° (decomp.) with preliminary softening at 205° which could not be eliminated by repeated recrystallization. (Found: C, 73·8, 73·8; H, 9·7, 9·2; CH_3CO, 15·5. $C_{34}H_{50}O_5$ (i.e. the diacetate of $C_{30}H_{46}O_4$) requires C, 73·6; H, 9·1; CH_3CO, 15·5; mol.wt. 554·8; $C_{34}H_{52}O_6$ requires C, 73·3; H, 9·4; CH_3CO, 15·5%, mol.wt. 556·8.)

The diacetate (28·05 mg.) in ethanol required 0·500 ml. of 0·1 N-NaOH for neutralization to phenolphthalein in the cold, indicating an equivalent of 561. $[\alpha]_D^{21°}$ in $CHCl_3 + 3·2°$ (*c*, 8·6). $[\alpha]_{5461}^{22°} + 4·2°$ in $CHCl_3$ (*c*, 6·0).

Both the monomethyl ester and the diacetate give immediate red-brown colorations with tetranitromethane indicating the presence of an olefinic double bond.

Ozonolysis of the diacetate. The diacetate (0·8 g.) in $CHCl_3$ (25 ml.) was treated with ozonized oxygen at room temperature for 3 hr. The ozonide was decomposed by warming the $CHCl_3$ solution with water at 60° whilst a current of nitrogen was passed through the liquid into a bubbler containing Brady's reagent (Brady & Elsmie, 1926). No volatile

carbonyl compound was detected in the issuing gas. The layers were separated and the $CHCl_3$ was washed with water, the washings being added to the water layer. Treatment of the latter with Brady's reagent (250 ml.) gave immediate yellow turbidity and flocculation. The precipitate was collected and dried. The crude product (160 mg., m.p. 155–160°) was crystallized from light petroleum and then from methanol giving yellow-orange needles of m.p. 164°, not depressed on admixture with authentic formaldehyde 2:4-dinitrophenylhydrazone of m.p. 164°. (Found C, 40·2; H, 3·2; N, 26·0. Calc. for $C_7H_6N_4O_4$: C, 40·0; H, 2·9; N, 26·7%.) The weight of crude material recovered represents a yield of 53% of theoretical for one methylene group. The $CHCl_3$ layer was not further examined.

Hydrogenation of triterpenoid acid

The acid (0·5000 g.), m.p. 285–290°, dissolved in ethanol (100 ml.), was hydrogenated at room temperature and ordinary pressure in the presence of Adams's PtO_2 catalyst (0·1000 g.). The corrected uptake of hydrogen was 24·6 ml. at N.T.P. in 1 hr. (uptake of 2H requires 23·8 ml.). The mixture was then filtered and the precipitated product washed out of the catalyst by warm ethanol. On evaporation a crude product (0·495 g., m.p. 295–297° (decomp.) was obtained, which on recrystallization from acetic acid gave microcrystals (70 mg.), elongated plates or flattened needles which began to darken at ~275°, shrank at ~280°, softened at ~295° and melted with effervescence at 295–297° (decomp.) $[\alpha]_D^{20°}$ in pyridine + 13·1° (*c*, 6·1). (Found: C, 75·8, 75·8; H, 10·2, 10·1. $C_{30}H_{48}O_4$ requires C, 76·3; H, 10·2; $C_{30}H_{50}O_4$ requires C, 75·9; H, 10·6%.)

Ungulinic acid

Anhydrous disodium salt. The crystalline salt obtained from the bicarbonate extract was dried in a vacuum desiccator (water-pump vacuum) over H_2SO_4 to constant weight and then exposed to a high vacuum at room temperature. No further loss in weight occurred. When the drying was continued at 55–60° in high vacuum, 98·89 mg. of the salt lost 4·01 mg., i.e. 4·05%, before attaining constant weight. $C_{22}H_{36}O_6Na_2 . H_2O$ requires 3·91% water. The anhydrous salt is hygroscopic.

Equivalent of the anhydrous salt

By sulphated ash. The disodium salt (94·29 mg.) when ashed with H_2SO_4 gave 29·33 mg. of Na_2SO_4. The Na content of 10·1% indicates a mol.wt. 456 for an anhydrous disodium salt. $C_{22}H_{36}O_6Na_2$ requires Na 10·4%, mol.wt. 442·5.

By back titration. The anhydrous disodium salt (90·61 mg.), after refluxing for 1 hr. with 0·1N-NaOH (5·02 ml.) with exclusion of CO_2, required 2·99 ml. of 0·1 N-HCl for neutralization to phenolphthalein. This corresponds with an apparent equivalent of 446, or a mol.wt. of 446 for a monoacid salt. Passage of CO_2 through the neutralized hydrolysate resulted in the almost immediate precipitation of a white crystalline salt. The reaction mixture was kept overnight in the refrigerator, and the precipitate was collected, washed and dried finally, in high vacuum at 55–60°; wt. 80 mg.

Monosodium salt

A sulphated ash determination showed that this was a monosodium salt; 79·06 mg. of the anhydrous salt afforded 14·05 mg. of Na_2SO_4; Na content 5·79%.

$C_{22}H_{37}O_6Na$ requires 5·47% Na. Decomposition of this salt with dil. HCl in the presence of ether gave the parent acid, m.p. 78–80° with slight previous softening. This acid has been named *ungulinic acid.*

Preparation and properties of the derived acids

Ungulinic acid anhydride, m.p. 53–54·5°

The hydrated disodium salt (0·50 g.) was dissolved in water by warming to about 50°. The solution frothed easily in the manner of a soap solution. After cooling to room temperature the solution was acidified with 2N-HCl, dropwise, until acid to congo red. A white flocculent precipitate was formed. The mixture was chilled overnight and the precipitate was collected and dried over H_2SO_4 to yield 0·36 g. of a crude acid of fatty appearance. On recrystallization from light petroleum (b.p. 60–80°) it formed colourless, thin, elongated plates, m.p. 53–54·5°. This substance, which we regard as the acid anhydride, is more stable than ungulinic acid. It is very soluble in all the common organic solvents. (Found: C, 69·3; H, 9·4; OCH_3, nil. $C_{22}H_{36}O_5$ requires C, 69·4; H, 9·5; mol.wt. 380·5; $C_{22}H_{38}O_5$ requires C, 69·1; H, 10·0%; mol.wt. 382·5.)

Equivalent. The acid anhydride (79·97 mg.), dissolved in ethanol (5 ml.), required 4·61 ml. of 0·1N-NaOH for neutralization to phenolphthalein, corresponding to an equivalent of 173·4. $C_{22}H_{36}O_5$ as dibasic acid requires 190·3, as tribasic acid, 126·8. Further NaOH (5·00 ml. of 0·1N) was then added to the neutralized solution and the mixture was refluxed for 1·75 hr. at 100°. The solution now required 3·25 ml. of 0·1 N-HCl for neutralization, indicating a further uptake of 1·75 ml. of alkali. The total alkali required is thus 6·36 ml. of 0·1N, indicating an equivalent of 126. Ungulinic acid anhydride thus behaves as a tribasic acid when treated with excess of hot alkali, but in the cold the equivalent is intermediate between that of a di- and a tri-basic acid.

After removal of the ethanol *in vacuo* the acid anhydride was precipitated by HCl. It had m.p. 51–52°, which was raised to 53–54° by recrystallization from light petroleum (wt. 60 mg.). It did not depress the melting point of a sample of the acid anhydride, m.p. 53–54·5°, which was the original material.

Molecular weight. The mol.wt. of the acid anhydride as determined by the cryoscopic method in dioxan was 358. $C_{22}H_{36}O_5$ requires 380·5.

Ungulinic acid, m.p. 78–80°

The hydrated salt (0·30 g.) was suspended in ether (100 ml.) and shaken with 2N-HCl (10 ml.), when the liberated acid passed into the ether layer. Evaporation of the ether left a white solid (0·25 g., m.p. 74·76°), which when recrystallized from ether-light petroleum (b.p. 40–60°) gave microcrystalline needles, m.p. 78–80°, after previous slight softening at 76°. The melt reset on cooling and remelted at 39–45°. The acid was less soluble in organic solvents than the anhydride, particularly in ether and light petroleum. The melting point of the acid, kept in a corked tube for 6 weeks, had fallen to 55–65°. (Found on the fresh sample: C, 65·3, 65·7, 65·1; H, 9·3, 9·3, 9·4. $C_{22}H_{38}O_6$ requires C, 66·3; H, 9·6. $C_{22}H_{38}O_6 . \frac{1}{4}H_2O$ requires C, 65·6; H, 9·6%.)

Optical activity. The acid, m.p. 78–80° (1·150 g.) in ethanol (30·0 ml.) was examined in a 4 dm. tube. The observed optical rotation was zero. Taking the maximum reading error as 0·01° the value for $[\alpha]_{5461}^{21°}$ is 0° within ±0·07°.

Dimethyl ester. The crystalline acid (0·1 g.) on treatment with an excess of diazomethane in ether quickly dissolved, with effervescence. The solution was left overnight and then the ether and excess reagent were removed *in vacuo* leaving a very pale-yellow viscous oil (0·12 g.). This was soluble in all the commoner organic solvents but could not be obtained crystalline. It was analysed after drying for 12 hr. in high vacuum at 40°. (Found: C, 67·7; H, 9·7; OMe, 14·8. $C_{24}H_{42}O_6$ (the dimethyl ester of $C_{22}H_{38}O_6$) requires C, 67·6; H, 9·9; OMe, 14·3%.) The dimethyl ester does not give the acetic anhydride-acetone colour reaction described for the parent acid.

Trihydrazide. The dimethyl ester obtained from 0·20 g. of the crystalline acid as above was dissolved in ethanol (3 ml.), treated with aqueous hydrazine hydrate (50% w/v; 1 ml.) and allowed to stand at room temperature. After a few hours a flocculent precipitate began to separate. After 24 hr. the precipitate was collected by filtration. The crude hydrazide (0·11 g.) was a white powder, m.p. 170–175°, which resisted all attempts at recrystallization. After refluxing several times with ethanol and drying over P_2O_5, it had m.p. 190–195° (decomp.). (Found: C, 59·8; H, 10·3; N, 17·8, 19·9. $C_{22}H_{44}O_3N_6$, the trihydrazide of $C_{22}H_{38}O_6$, requires C, 60·0; H, 10·1; N, 19·1%.)

Colour reactions of ungulinic acid and anhydride

Both acid and anhydride give the following colour reactions:

When warmed with acetic anhydride at 100° for a few minutes a pink colour develops, which on addition of excess acetone gives rise to a pronounced green fluorescence.

When dissolved in a few drops of pyridine and treated with a few drops of acetic anhydride in the cold a deep-red colour is produced with a pronounced green fluorescence. On slight warming the colour changes to a dark brown-violet, retaining the green fluorescence. These reactions are also given by agaricic acid.

Interconversion of ungulinic acid and its anhydride

Conversion of ungulinic acid, m.p. 78–80°, to the anhydride, m.p. 53–54·5°. The acid was quickly dried to constant weight in a vacuum desiccator. When further dried in a high vacuum at 48–52°, 102·27 mg. lost 4·47 mg. before the weight again became constant. The original material gradually passed to a pale-brown, effervescent mass, which eventually ceased to effervesce; this weighed 97·8 mg. If this loss corresponds to 1 mol. of water the mol.wt. of the acid would be 412. $C_{22}H_{38}O_6$ requires mol.wt. 398·5. The melting point of the dried material was 42–46°, but recrystallization from light petroleum (b.p. 40–60°) raised it to 53–54°, undepressed on admixture with the anhydride of m.p. 53–54·5°.

Conversion of the anhydride to ungulinic acid. The anhydride (0·15 g.) was dissolved in N-Na_2CO_3 (15 ml.) and filtered to remove a slight turbidity. CO_2 was passed through the clear filtrate giving a crystalline Na salt (0·12 g.).

Regeneration of the acid by shaking the salt with ether in presence of 2N-HCl gave 0·10 g. of product of m.p. 74–76°, raised by recrystallization from light petroleum to 78–80° (with previous slight softening). The melting point was undepressed on admixture with a sample of ungulinic acid of the same melting point, prepared independently.

Potassium hydroxide fusion of the disodium salt

The disodium salt (0·55 g.) of ungulinic acid was heated with KOH (2·5 g.) and water (1·5 ml.). As the temperature rose effervescence occurred at 190–200° and again at about 290°. The temperature was maintained at 310° for 1 hr. The reaction mixture after cooling was treated with water. The product did not dissolve readily, but was replaced by a flocculent precipitate which passed slowly into solution on warming. The solution was clarified by filtration through a sintered-glass filter and the filtrate was acidified, dropwise, with H_2SO_4 (30% v/v) to give an almost white flocculent precipitate (0·31 g.) of m.p. 55–60°. Several recrystallizations from light petroleum (b.p. 40–60°) raised the melting point to 65–67°, and gave a product of colourless shining leaflets. (Found: C, 76·1; H, 12·4, equivalent by microtitration 274. $C_{18}H_{36}O_2$ requires C, 76·0; H, 12·8, equivalent (monobasic) 284.)

This product did not depress the melting point of an authentic sample of stearic acid, m.p. 66–68°; when mixed with palmitic acid, the sample produced a definite depression.

The acidified filtrate was evaporated repeatedly *in vacuo* to remove any volatile acids. The aqueous distillate required 9·45 ml. of 0·1N-NaOH for neutralization. The solution of Na salt was evaporated to small volume (4 ml.) and refluxed with *p*-bromophenacyl bromide (0·28 g.) in ethanol (6 ml.) for 2 hr. The reaction mixture deposited crystals (80 mg., 70–75°) on cooling which when recrystallized from light petroleum had m.p. 83–85° and did not depress the m.p. (84–85°) of an authentic sample of *p*-bromophenacyl acetate.

The acidified concentrated aqueous solution containing the non-volatile material was continuously extracted with ether (14 hr.) to give a further quantity of crystalline material (0·10 g.). This was dissolved in water, neutralized and the oxalic acid precipitated with aqueous $CaCl_2$ as calcium oxalate (0·13 g.). No other acid was detectable after removal of the oxalic acid.

Potassium hydroxide fusion of ungulinic acid (disodium salt) thus gives rise to stearic, acetic and oxalic acids.

All melting points in this paper are uncorrected.

SUMMARY

1. The metabolic products of the wood-rotting fungus *Polyporus benzoinus* (Wahl.) Fr., when cultivated on laboratory media, have been examined.

2. Anisaldehyde, isolated as the 2:4-dinitrophenylhydrazone, is probably the main contributor to the aromatic odour.

3. A triterpenoid acid, $C_{30}H_{46}O_4$ (or $C_{30}H_{48}O_4$), m.p. 285–290° (decomp.), $[\alpha]_{5461}^{20°}$ + 8·19° in pyridine, was isolated. This is a dihydroxymonobasic acid with a terminal methylene group. It appears to be identical with polyporenic acid *C*, obtained by Cross *et al.* (1940) from the natural sporophore of *P. betulinus*.

4. A second acid, ungulinic acid, probably $C_{22}H_{38}O_6$, m.p. 78°, was obtained from the metabolic products. This acid gives a more stable anhydride

$C_{22}H_{36}O_5$, m.p. 53–54·5°. The acid is potentially tribasic; it appears to have two carboxyl groups and one lactonic grouping. It affords stearic, oxalic and acetic acids on potassium hydroxide fusion. Its structure is not determined, but a hydroxytricarballylic structure with a normal C_{16} alkyl chain as substituent is tentatively suggested.

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APPENDIX

Antibacterial activity of the triterpenoid acid (polyporenic acid C) and of ungulinic acid, metabolic products of *Polyporus benzoinus* (Wahl.) Fr.

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METHODS

For the determination of antibacterial activity the sodium salts of the respective acids were used for preparing a series of dilutions in broth which were inoculated with the test organisms, incubated at 37° and observed after a suitable interval for growth, and in the case of the Mycobacteria, for pellicle formation. The dilutions were made as follows:

Triterpenoid acid. The purified acid of m.p. 285–290° (decomp.) (10 mg.) was dissolved in ethanol (5 ml.) with slight warming. The equivalent amount of 0·1 N-NaOH (0·255 ml.) was then added and the solution of the sodium salt was diluted to 10 ml. to give a 1:1000 solution and from this the desired twofold dilutions in Lab-Lemco-peptone broth +0·2% glucose, pH 7·5, were prepared, starting at 1:10,000.

Ungulinic acid. The hydrated disodium salt of the acid was used. A 1:1000 solution was obtained by dissolving the salt (30·0 mg.) in glucose broth (30 ml.) with careful warming to 40–50°. From this solution twofold dilutions in broth were prepared, starting at 1:2000.

The serial dilutions (4·5 ml. quantities in $6 \times \frac{5}{8}$ in. test tubes) of the two substances were inoculated with one loopful of an undiluted culture of the micro-organisms used, viz. *Staphylococcus aureus*, Oxford H; *Bacillus coli*, N.C.T.C. 86; *Mycobacterium phlei*, N.C.T.C. 525; and *M. smegmatis*, N.C.T.C. 523.

RESULTS

The triterpenoid acid (Table 1) was found to have a relatively low antibacterial activity against *Staph. aureus* and *B. coli* (24 hr.) whereas the activity against the Mycobacteria was high, no growth being obtained with *M. phlei* after 72 hr. with a dilution of 1:1,280,000 of the antibiotic. In this case the activity disappeared on further incubation up to 7 days. The ungulinic acid (Table 2), on the other hand, showed a moderate activity against *Staph. aureus* (1:128,000), none against *B. coli* (24 hr.) and only low activity against *M. phlei* (1:8000) after 77 hr., which, however, was only slightly reduced in 7 days.

An attempt was made to determine the antibacterial action of the two metabolic products

Table 1. *Antibacterial activity of polyporenic acid C (as sodium salt)*

(Symbols: −, complete inhibition; ±, slight growth; +, moderate growth; + +, full growth; P, pellicle formation.)

Organism	Growth observed after	Dilutions 1 in 10,000	20,000	40,000	80,000	160,000	320,000	640,000	1,280,000	2,560,000
Staph. aureus	24 hr.	+	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
B. coli	24 hr.	−	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. phlei	72 hr.	−	−	−	−	−	−	−	−	+ + P
M. phlei	7 days	+	+ + P	.	.	.	.	.	.	.
M. smegmatis	72 hr.	−	−	−	−	−	−	−	+ + P	+ + P
M. smegmatis	7 days	+ + P	.	.	.	.	.	.	.	.

Table 2. *Antibacterial activity of ungulinic acid (as disodium salt)*

(Symbols, as in Table 1.)

Organism	Growth observed after	Dilutions 1 in 2000	4000	8000	16,000	32,000	64,000	128,000	256,000
Staph. aureus	24 hr.	−	−	−	−	−	−	−	+ +
B. coli	24 hr.	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
M. phlei	77 hr.	−	−	−	± P	+ P	+ + P	+ + P	+ + P
M. phlei	7 days	−	−	+ + P	.	.	.	.	.

against *M. phlei* and *M. smegmatis*, using the medium of Dubos (1947), but the results were unsatisfactory, since the lower dilutions showed a turbidity which was undistinguishable from the growth of the organism. A point of minimum turbidity was noted however in each case, which may give some indication of the limiting dilution. This occurred with the triterpenoid acid at 1:16,000 and with ungulinic acid at 1:4000 for both species of Mycobacteria.

SUMMARY

1. The triterpenoid acid, polyporenic acid *C*, is a powerful antibacterial substance against Mycobacteria, but has little activity against *Staphylococcus aureus* and *Bacillus coli*. Even this high activity against Mycobacteria disappears on prolonged incubation.

2. Ungulinic acid is moderately active only against *Staph. aureus* of the organisms tested.

REFERENCE

Dubos, R. J. (1947). *Experientia, Basel*, **3**, 45.

The Composition of the Depot Fats of a Pig Fed on a Diet Rich in Whale Oil

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The facility with which the pig can utilize dietary fat for the purpose of its own reserve fat has been recognized for many years. Thus, Ellis & Isbell (1926) showed that the depot fats of pigs fed respectively on soya beans and on ground nuts were markedly different from those of pigs fed on a normal (corn) diet low in fat and contained unusually large proportions of linoleic acid and minor proportions of linolenic acid or of arachidic and higher saturated acids, which are present in the respective fatty oils of the soya bean or the ground nut. Similar results were observed by Ellis, Rothwell & Pool (1931) when cotton-seed oil was added to the normal diet of pigs. When a diet which included 14% of a fish (menhaden) oil was given to pigs, Brown (1931) observed that the pig body fat contained small but significant proportions of the unsaturated acids of the C_{20} and C_{22} series which are characteristic components of the fish oil.

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An exceptionally favourable opportunity to study this matter in further detail recently occurred when specimens of the adipose tissues of a pig which had been reared on a diet exceptionally rich in whale oil at the Animal Research Station, Cambridge, were placed at our disposal by the Director of Food Investigation, Dr F. Kidd, F.R.S. The animal had been fed from weaning on a diet which contained 50 % of crude whale oil and in the final stages of fattening was receiving 4 lb. of whale oil per day. At slaughter (at 198 days) it weighed over 200 lb., its rate of growth being normal and its condition apparently fully healthy. The fat depots after slaughter were found to have a yellowish brown coloration and to present certain abnormal features which led Mr J. K. Walley (who was carrying out the investigation) to consult us. The average unsaturation (iodine value) of the depot fats was abnormally high and the specimens appeared to merit a quantitative examination of their component acids and glycerides, which we readily agreed to undertake and the results of which are here recorded.

METHODS AND RESULTS

Specimens of the perinephric, inner back and outer back adipose tissues sent to us were extracted exhaustively with acetone. The matter extracted by acetone was taken up in light petroleum (b.p. 40–60°), and the solution filtered from insoluble matter, after which the lipid matter from each tissue was separately recovered and found to have the general characteristics shown in Table 1.

Table 1. *Analytical characteristics of the pig depot fats*

	Source of fat		
	Perinephric	Inner back	Outer back
Iodine value	83·7	85·7	86·5
Saponification equivalent	286·4	287·8	286·0
Free fatty acid (as oleic, %)	0·6	0·9	0·4
Unsaponifiable matter (%)	1·2	0·2	0·8
Peroxide value	0·3	4·8	5·1

Component fatty acids of the pig fats

The mixed acids from about 120 g. of each fat were examined quantitatively by preliminary resolution into three (or four) groups by crystallization, first from acetone at −60°, the deposited acids (in one instance after a recrystallization from acetone at −60°) being then crystallized from ether at −30°. The methods used for the fractional distillation of the methyl esters of each group of acids and for the analytical (including spectrophotometric) examination of the ester fractions, were those described (Gupta & Hilditch, 1951). In order, however, to illustrate the typically 'marine animal' nature of the acids left in solution in acetone at −60°, the ester fractionation data for the groups of acids from all three depot fats are recorded in Table 3. The results of the solvent separations of the mixed fatty acids are summarized in Table 2.

Table 2. *Low temperature crystallization of the mixed acids from each pig depot fat*

Solubility characteristics of fraction	Weight (g.)	Weight (% of total)	Iodine value
Perinephric fat			
A. Insoluble in ether at −30°	39·0	35·5	3·1
B. Soluble in ether at −30°	34·4	31·3	77·7
C. Soluble in acetone at −60°	36·5	33·2	177·1
Inner back fat			
A. Insoluble in ether at −30°	31·6	30·2	2·8
B. Soluble in ether at −30°	26·9	25·7	78·0
C. Soluble in acetone at −60°	45·9	44·1	148·3
Outer back fat			
A. Insoluble in ether at −30°	29·9	27·9	6·2
B. Soluble in ether at −30°	42·7	39·9	83·9
C. Insoluble in acetone at −60° (recrystallization)	9·2	8·6	148·2
D. Insoluble in acetone at −60° (first crystallization)	25·3	23·6	175·8

The composition of each ester fraction was then calculated by methods described (Hilditch, 1947, pp. 498–510; Gupta & Hilditch, 1951), and therefrom the percentage composition (by wt.) of each group of acids from each fat. These compositions and the consequent composition of the total fatty acids in each of the three depot fats are shown in Table 4.

Glyceride composition of the pig perinephric fat

The figures in Table 4 show clearly that all three depot fats of the pig which had received a diet rich in whale oil contained considerable amounts of the highly unsaturated acids characteristic of whale oil and, indeed, that these were of much the same average unsaturation as those in a typical whale oil (cf. Table 6). It was therefore decided to see whether, by suitable crystallization procedure, one of the fats could be separated roughly into fractions which would resemble respectively whale oil and the type of fat which the pig is known to elaborate from a non-fatty diet. It is known (Hilditch & Maddison, 1948) that all but about 20 % of the (more saturated) glycerides in Antarctic whale oil remain in solution in acetone at −40°, whereas only a very small percentage of pig depot fat glycerides from animals on a diet low in fat are soluble in acetone at this temperature.

A quantity of the pig perinephric fat was therefore crystallized from a 10 % solution in acetone at −40°, the deposited glycerides being recrystallized under the same conditions and the procedure repeated until only a very small amount of glycerides remained in solution. In this way the following fractions were successively obtained:

	Weight (g.)	Iodine value
First crystallization	38·7	163·5
Second crystallization	15·7	136·0
Third crystallization	7·7	126·0
Fourth crystallization	2·6	122·0
Fifth crystallization	2·5	112·4
Glycerides deposited (fifth crystallization)	191·9	59·7

Table 3. *Fractionation data obtained with esters of fatty acids from depot fats of pig*

(The solubility characteristics of fractions *A*, *B*, *C* and *D* are given in Table 2.)

Fraction	Weight (g.)	Sapon. equiv.	Iodine value	Fraction	Weight (g.)	Sapon. equiv.	Iodine value
	Perinephric fat				Inner back fat		
Methyl esters of acids *A*:				Methyl esters of acids *A*:			
*A*1	2·17	255·0	0·4	*A*1	2·09	265·9	1·2
*A*2	3·81	265·9	0·6	*A*2	3·19	270·4	1·2
*A*3	4·40	269·6	0·4	*A*3	3·67	271·0	1·5
*A*4	4·18	270·1	0·7	*A*4	4·08	273·3	1·7
*A*5	4·24	271·5	0·9	*A*5	4·28	275·7	2·5
*A*6	5·47	281·3	5·1	*A*6	4·09	278·7	3·0
*A*7	8·25	294·9	6·4	*A*7	3·61	286·9	4·3
*A*8	2·61	297·0	6·5	*A*8	4·22	288·2	4·8
*A*9	2·34	300·4*	8·2	*A*9	2·91	294·7†	8·2
Total	37·47	—	—	Total	32·14	—	—
Methyl esters of acids *B*:				Methyl esters of acids *B*:			
*B*1	1·40	247·8	10·8	*B*1	2·46	268·1	38·5
*B*2	1·83	268·5	33·8	*B*2	2·94	287·1	67·4
*B*3	3·35	286·7	66·1	*B*3	3·66	288·0	69·2
*B*4	3·03	291·5	75·5	*B*4	4·31	292·7	79·0
*B*5	3·39	293·2	75·8	*B*5	3·75	295·8	80·2
*B*6	2·99	293·6	79·6	*B*6	3·29	295·7	81·6
*B*7	3·99	294·8	79·5	*B*7	4·27	296·8	83·5
*B*8	3·02	295·3	80·8	*B*8	3·00	300·5†	87·8
*B*9	3·27	295·4	81·5	Total	27·58	—	—
*B*10	3·16	296·8	82·7				
*B*11	2·63	303·9*	87·1				
Total	32·06	—	—				
Methyl esters of acids *C*:				Methyl esters of acids *C*:			
*C*1	1·14	205·6	47·6	*C*1	1·95	242·8	38·5
*C*2	2·48	261·3	86·0	*C*2	4·11	260·7	91·2
*C*3	3·90	273·5	102·0	*C*3	3·24	277·2	100·8
*C*4	2·98	288·7	119·3	*C*4	4·82	288·9	108·1
*C*5	3·40	287·6	120·0	*C*5	4·65	291·7	111·0
*C*6	3·27	289·7	126·8	*C*6	5·96	295·6	110·5
*C*7	3·78	293·4	133·6	*C*7	5·38	296·0	114·0
*C*8	3·08	301·4	185·0	*C*8	4·47	298·0	121·9
*C*9	3·19	303·2	193·4	*C*9	4·15	301·4	149·4
*C*10	3·45	307·2	219·3	*C*10	4·15	314·5	239·4
*C*11	3·71	325·4	334·0	*C*11	3·88	333·1†	300·8
*C*12	2·19	359·8*	257·0	Total	46·76	—	—
Total	36·57	—	—				
				Outer back fat			
Methyl esters of acids *A*:				Methyl esters of acids *C*:			
*A*1	3·24	254·0	1·2	*C*1	1·63	267·3‡	83·6
*A*2	2·50	269·4	1·0	*C*2	1·80	282·0	111·5
*A*3	3·03	270·0	2·0	*C*3	1·90	291·7	123·1
*A*4	3·67	274·3	4·4	*C*4	1·37	299·2	153·7
*A*5	4·50	275·9	5·2	*C*5	1·24	306·4	178·7
*A*6	2·78	275·2	5·6	*C*6	1·06	351·3‡	232·0
*A*7	3·15	285·3	10·3	Total	9·00	—	—
*A*8	3·48	291·7	11·1				
*A*9	3·44	297·8‡	14·8				
Total	29·79	—	—				
Methyl esters of acids *B*:				Methyl esters of acids *D*:			
*B*1	1·78	238·4	30·5	*D*1	1·22	249·4‡	67·8
*B*2	2·42	274·3	54·4	*D*2	2·12	267·3	90·8
*B*3	3·53	286·1	65·6	*D*3	2·27	268·9	108·3
*B*4	2·93	287·1	67·7	*D*4	2·30	284·5	128·7
*B*5	4·08	296·4	82·8	*D*5	2·23	286·0	128·6
*B*6	4·17	296·2	84·4	*D*6	2·79	289·5	134·3
*B*7	5·73	297·8	83·8	*D*7	3·06	293·8	143·7
*B*8	5·57	298·6	85·3	*D*8	2·17	296·6	168·2
*B*9	4·30	300·4	85·2	*D*9	2·57	307·4	212·5
*B*10	4·76	308·4‡	87·9	*D*10	2·14	319·2	272·8
*B*11	2·69	317·4‡	101·9	*D*11	2·55	328·3‡	303·9
Total	41·96	—	—	Total	25·42	—	—

* Equivalents of esters (freed from unsaponifiable matter): *A*9, 297·6; *B*11, 297·1; *C*12, 333·2.

† Equivalents of esters (freed from unsaponifiable matter): *A*9, 288·5; *B*8, 300·5; *C*11, 329·2.

‡ Equivalents of esters (freed from unsaponifiable matter): *A*9, 294·1; *B*10, 301·4; *B*11, 308·0; *C*1, 263·1; *C*6, 342·5; *D*1, 248·3; *D*11, 328·3.

Table 4. *Component acids in groups A, B, C (D), and in the whole pig depot fats*

(Separation into fractions *A*, *B*, *C* and *D* is described in Table 2.)

Acid	*A* (%)*	*B* (%)*	*C* (%)*	*D* (%)*	Fatty acids in the whole fat (%, w/w)	Fatty acids in the whole fat (%, by mol.)
			Perinephric fat			
	(35·5 %)†	(31·3 %)†	(33·2 %)†	—		
Myristic acid	4·5	3·1	1·9	—	3·3	4·1
Palmitic acid	55·3	9·7	4·7	—	24·2	25·8
Stearic acid	36·2	2·0	—	—	13·5	12·9
Unsaturated C_{14}	—	0·3	2·7	—	1·0	1·2
Unsaturated C_{16}	0·3	3·4	15·1	—	6·2	6·7
Unsaturated C_{18}	3·7	80·7	48·6	—	42·8	41·4
Unsaturated C_{20}	—	0·6	20·3	—	7·0	6·2
Unsaturated C_{22}	—	—	6·3	—	2·0	1·7
Unsaponifiable	Trace	0·2	0·4	—	—	—
Mean unsaturation‡ of:						
C_{14} acids	—	−2·0	−2·0	—	−2·0	
C_{16} acids	−2·0	−2·0	−2·1	—	−2·1	
C_{18} acids	−2·0	−2·0	−3·3	—	−2·5	
C_{20} acids	—	−3·0	−7·3	—	−7·2	
C_{22} acids	—	—	−10·3	—	−10·3	
			Inner back fat			
	(30·2 %)†	(25·7 %)†	(44·1 %)†	—		
Myristic acid	0·9	0·3	2·8	—	1·6	1·9
Palmitic acid	62·2	11·2	2·4	—	22·7	24·3
Stearic acid	33·1	2·5	—	—	10·6	10·3
Unsaturated C_{14}	—	—	3·2	—	1·4	1·7
Unsaturated C_{16}	0·4	5·6	13·2	—	7·4	8·0
Unsaturated C_{18}	3·2	78·0	59·4	—	47·3	45·8
Unsaturated C_{20}	—	2·4	15·7	—	7·6	6·8
Unsaturated C_{22}	—	—	3·2	—	1·4	1·2
Unsaponifiable	0·2	—	0·1	—	—	—
Mean unsaturation‡ of:						
C_{14} acids	—	—	−2·0	—	−2·0	
C_{16} acids	−2·0	−2·0	−2·1	—	−2·1	
C_{18} acids	−2·0	−2·0	−2·7	—	−2·4	
C_{20} acids	—	−3·0	−6·5	—	−6·2	
C_{22} acids	—	—	−8·5	—	−8·5	
			Outer back fat			
	(27·9 %)†	(39·9 %)†	(8·6 %)†	(23·6 %)†		
Myristic acid	5·9	3·0	2·9	1·6	3·5	4·2
Palmitic acid	58·5	6·1	3·0	2·0	19·5	20·8
Stearic acid	28·4	0·8	—	—	8·2	7·9
Unsaturated C_{14}	—	1·2	—	2·0	0·9	1·2
Unsaturated C_{16}	1·8	3·5	23·4	24·1	9·7	10·4
Unsaturated C_{18}	5·3	75·4	49·3	45·5	46·7	45·4
Unsaturated C_{20}	—	9·5	11·2	18·6	9·2	8·2
Unsaturated C_{22}	—	—	9·5	6·2	2·3	1·9
Unsaponifiable	0·1	0·5	0·7	Trace	—	—
Mean unsaturation‡ of:						
C_{14} acids	—	−2·0	—	−2·0	−2·0	
C_{16} acids	−2·0	−2·0	−2·1	−2·1	−2·1	
C_{18} acids	−2·0	−2·0	−3·1	−3·4	−2·4	
C_{20} acids	—	−3·0	−5·2	−6·8	−5·1	
C_{22} acids	—	—	−6·4	−8·8	−7·9	

* Component acids as % (w/w) of group.

† Groups as % (w/w) of total acids.

‡ Mean unsaturation expressed as number of hydrogen atoms necessary to convert 1 mol. of acid to the saturated state (Hilditch, 1947, p. 23).

The portions left in solution were mixed and the fatty acids in the two groups so obtained were analysed in detail by the methods described above. There were thus obtained:

	Weight (g.)	Weight (% of total)	Iodine value
Glycerides insoluble in acetone at $-40°$	191·9	74·1	59·7
Glycerides soluble in acetone at $-40°$	67·2	25·9	149·6

The final results of the component acid analyses of each group are summarized in Table 5.

Table 5. *Component fatty acids (%, w/w) of the pig perinephric glycerides separated by crystallization from acetone at $-40°$*

Acid	Insoluble in acetone at $-40°$	Soluble in acetone at $-40°$
Myristic acid	2·1	1·5
Palmitic acid	25·7	14·3
Stearic acid	16·5	1·4
Unsaturated C_{14}	0·3	0·8
Unsaturated C_{16}	5·4	11·1
Unsaturated C_{18}	43·5	44·3
Unsaturated C_{20}	6·5	21·2
Unsaturated C_{22}	—	5·4
Mean unsaturation of:		
C_{14} acids	−2·0	−2·0
C_{16} acids	−2·1	−2·1
C_{18} acids	−2·3	−2·9
C_{20} acids	−6·4	−6·8
C_{22} acids	—	−10·3

Comparison of the figures in Table 5 with those of a typical perinephric fat from a pig fed on a low-fat diet and of a typical Antarctic whale oil (Table 6) shows that the fat under investigation had been largely resolved by the physical process of crystallization into pig synthetic fat and the assimilated dietary fat.

DISCUSSION

The figures in Table 4 not only demonstrate the marked extent to which the component acids of the three depot fats differ from those of a pig fed on a diet low in fat, but suggest that the component acids of whale oil are present in significant amounts and that, moreover, the composition of these abnormal pig depot fats might be explained on an additive basis: they appear, prima facie, to be a mixture of typical pig depot fat (as synthesized from carbohydrate) with more or less unaltered whale oil. This tentative conclusion is reinforced by more detailed examination of the fatty acid compositions observed in each of the three fats.

Fortunately, it is well established that the respective compositions of perinephric, inner and outer back fats from pigs fed on diets low in fat are remarkably uniform (Ellis & Isbell, 1926; Ellis & Zeller, 1930; Ellis *et al.* 1931; Hilditch, Lea & Pedelty, 1939; Hilditch & Pedelty, 1940), and consequently figures for such biosynthesized fats are available. In addition, although the specimen of Antarctic whale oil fed to the pigs was not analysed in detail, the component fatty acids of Antarctic whale oils have been found to vary but little over a range of several seasons (Armstrong & Allan, 1924; Tveraaen, 1935; Hilditch & Maddison, 1948). Typical figures for the component acids of depot fats of pigs fed on a low-fat diet (Hilditch & Pedelty, 1940) and of Antarctic whale oil (Hilditch & Maddison, 1948) are given in Table 6.

The palmitic acid contents of all the fats in Table 6 are highly characteristic. In a mixture of pig fat and whale oil, therefore, the approximate proportions of each of these fatty oils could be deduced from the percentage of palmitic acid in the total fatty acids of the mixture (Table 7).

The fatty acid compositions of mixtures of the respective depot fats (Table 6) with Antarctic whale

Table 6. *Component acids (%, w/w) of depot fats of pigs (on low-fat diet) and component acids of Antarctic whale oil*

Acid	Pig depot fats: Perinephric	Pig depot fats: Inner back	Pig depot fats: Outer back	Antarctic whale oil
Myristic acid	0·9	0·9	1·0	9·2
Palmitic acid	29·3	27·5	26·5	15·6
Stearic acid	17·4	15·1	12·8	2·5
Unsaturated C_{14}	0·3	0·2	0·2	2·5
Unsaturated C_{16}	1·8	1·7	1·9	13·9
Unsaturated C_{18}	48·4	51·5	54·7	37·2
Unsaturated C_{20} / Unsaturated C_{22}	1·9	3·1	2·9	12·0 / 7·1
Mean unsaturation of:				
C_{14} acids	−2·0	−2·0	−2·0	−2·0
C_{16} acids	−2·0	−2·0	−2·0	−2·1
C_{18} acids	−2·3	−2·3	−2·3	−2·4
C_{20} acids / C_{22} acids	−5·0	−5·0	−5·0	−7·1 / −9·4

oil in the proportions shown in Table 7 have been calculated and are compared in Table 8 with the results actually found for the three pig depot fats (Table 4). The close similarity between the 'calculated' figures and those found for each individual acid (including the mean unsaturation of the C_{20} and C_{22} acids present) appears to offer conclusive proof that the whale oil has been deposited in a purely additive manner alongside the fat synthesized by the pig. Moreover, in the outer layers of the back fat, which are invariably softer and more unsaturated than the inner layers or the perinephric fat, the increase in unsaturation appears to be produced by a marked increase in the proportion of deposited whale oil.

The figures in Table 8 can hardly be interpreted on any other basis than that whale oil, substantially unchanged, has passed along with the fat synthesized by the pig itself into the fat depots of the animal and, further, that the typical differences in unsaturation and consistency of fats in the different depots have been attained by variation in the pro-

Table 7. *Calculated proportions of (biosynthesized) pig fat and whale oil (calculated from the percentage of palmitic acid in the three depot fats)*

	Palmitic acid in depot fatty acids (%)	Calculated proportions of	
		True pig fat (%)	Whale oil (%)
Perinephric fat	24·2	62	38
Inner back fat	22·7	60	40
Outer back fat	19·5	36	64

Table 8. *Comparison of observed and 'calculated' compositions of the pig depot fats. (Component fatty acids, % w/w)*

(For details of calculation see text and Tables 6 and 7.)

	Source of fat					
	Perinephric		Inner back		Outer back	
Acid	Found	'Calculated' (62% pig: 38% whale)	Found	'Calculated' (60% pig: 40% whale)	Found	'Calculated' (36% pig: 64% whale)
Myristic acid	3·3	4·1	1·6	4·2	3·5	6·2
Palmitic acid	24·2	(24·2)	22·7	(22·7)	19·5	(19·5)
Stearic acid	13·5	11·7	10·6	10·1	8·2	6·2
Unsaturated C_{14}	1·0	1·1	1·4	1·1	0·9	1·7
Unsaturated C_{16}	6·2	6·4	7·4	6·6	9·7	9·6
Unsaturated C_{18}	42·8	44·1	47·3	45·8	46·7	43·5
Unsaturated C_{20}	7·0	5·3	7·6	6·0	9·2	8·4
Unsaturated C_{22}	2·0	3·1	1·4	3·5	2·3	4·9
Mean unsaturation of:						
C_{14} acids	−2·0	−2·0	−2·0	−2·0	−2·0	−2·0
C_{16} acids	−2·1	−2·0	−2·1	−2·0	−2·1	−2·1
C_{18} acids	−2·5	−2·3	−2·4	−2·3	−2·4	−2·4
C_{20} acids	−7·2	−7·0	−6·2	−7·0	−5·1	−7·0
C_{22} acids	−10·3	−9·0	−8·5	−9·0	−7·9	−9·0

Table 9. *Comparison of the solvent-segregated perinephric fat with normal pig perinephric fat and whale oil. (Component fatty acids, % w/w)*

		Present perinephric fat (Table 5)		
Acid	Typical pig perinephric fat	Insoluble portion	Soluble portion	Antarctic whale oil
Myristic acid	0·9	2·1	1·5	9·2
Palmitic acid	29·3	25·7	14·3	15·6
Stearic acid	17·4	16·5	1·4	2·5
Unsaturated C_{14}	0·3	0·3	0·8	2·5
Unsaturated C_{16}	1·8	5·4	11·1	13·9
Unsaturated C_{18}	48·4	43·5	44·3	37·2
Unsaturated C_{20}	1·9	6·5	21·2	12·0
Unsaturated C_{22}		—	5·4	7·1
Mean unsaturation of:				
C_{14} acids	−2·0	−2·0	−2·0	−2·0
C_{16} acids	−2·0	−2·1	−2·1	−2·1
C_{18} acids	−2·3	−2·3	−2·9	−2·4
C_{20} acids	−5·0	−6·4	−6·8	−7·1
C_{22} acids		—	−10·3	−9·4

portions of whale oil and synthesized pig fat laid down. This conclusion is reinforced by the resolution of the perinephric fat by the physical process of crystallization under appropriate conditions into 26 % of a fatty oil not very different from whale oil and 74 % of a fat closely resembling pig perinephric fat as synthesized in the animal (Table 9).

Under the conditions of crystallization some 20 % of the more saturated glycerides of any whale oil present would have been left in the insoluble fraction, so that the 26 % of soluble matter probably represents about 33 % of whale oil originally present. Exact reproduction respectively of a typical 'synthesized' pig fat or of whale oil by either segregate is therefore not to be expected, but the approach towards each oil is somewhat striking, especially in the palmitic and stearic acid contents of each portion. The presence of a small proportion of characteristic whale oil acyl groups in the insoluble portion is indicated by the larger proportions of unsaturated C_{16} and C_{20} acids as compared with a typical pig perinephric fat.

Since dietary fat is largely assimilated through the lymphatic system before it enters the blood stream, it is perhaps not unnatural that the depot fats of a pig fed heavily on whale oil should exhibit the compositions disclosed in the course of this study. Yet we believe that the conditions governing the deposition of dietary and synthesized fats have not previously been depicted so clearly as in the present instance, and we are glad to have had the opportunity to examine these especially informative specimens. It is, moreover, somewhat remarkable that the highly reactive unsaturated groups in the marine animal fat have undergone no perceptible alteration during their passage through the animal: nor, indeed, is there any indication of chemical interchange between the acyl groups of the fat synthesized in the pig and the whale glycerides. Had this taken place to any appreciable extent the physical resolution of the fat by solvent segregation could not have given the results which were in fact obtained. At first sight it might also be supposed that the present study throws further light on the processes involved in the passage of dietary fat into the lymphatic system, but this is not so. Since the whale oil was ingested in massive proportions, it would represent practically all the fat which was absorbed as such: consequently, its constituent glycerides would be substantially the same whether they were hydrolysed and resynthesized during passages from the intestine into the lymphatics, or whether they were translocated as such without hydrolysis. It would seem, however, that with a suitably modified fatty diet it should be possible to obtain some more conclusive evidence in this direction, and it is hoped that one of us may be able to undertake further studies to this end in the near future.

SUMMARY

1. The component acids present in the perinephric, inner back and outer back fats of a pig which had been fed heavily on whale oil have been examined. The results show that considerable proportions of the characteristic whale oil fatty acids had passed into the fat depots, especially in the case of the outer back fat.

2. Crystallization of the perinephric fat from acetone at $-40°$ separated it into 26 % of a soluble fraction which approximated in composition to whale oil, and left 74 % of insoluble material, the composition of which closely resembled a normal pig perinephric fat except for the presence of somewhat more unsaturated acids of the C_{16} and C_{20} series than are usually found in the latter.

3. The depot fats from the animal whose diet had included 50 % of whale oil therefore consist of a mixture of whale oil with the typical fat synthesized by the pig from non-fatty food. The compositions of the individual component acids in each of the three fats, when calculated on the assumption that they were mixtures of typical 'synthesized' depot fats with whale oil in appropriate proportions, agreed closely in most cases with those determined experimentally.

4. It was noticeable that the high degree of unsaturation of the acids of the C_{20} and C_{22} series in whale oil was reproduced in the corresponding fatty acids of the deposited fat. This emphasizes the manner in which the whale oil glycerides had been assimilated and deposited in the depots without any chemical alteration.

5. The greater unsaturation (leading to softer consistency) of the fat in the outer layers of the back adipose tissue was attained by a relatively higher proportion of deposited whale oil to typical synthesized pig fat than in the inner layers or perinephric fat.

We are greatly indebted to the Director of Food Investigation, Dr F. Kidd, F.R.S., and to Mr J. K. Walley of the Low Temperature Research Station, Cambridge, who drew our attention to these interesting specimens of pig depot fat and who have very kindly permitted us to make use of their material in the course of this investigation. We thank Mr R. E. Bridge, who assisted us by determining the component acids present in two of the pig fats.

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Observations on the Determination of the Specific Activity of the Inorganic Phosphate Fraction of Trichloroacetic Acid Extracts of Liver

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The determination of the specific activity (S.A.) of inorganic phosphorus* present in a tissue extract involves its quantitative separation from organophosphates and the subsequent measurement of the radioactivity. Measurement of inorganic P is made directly on another sample. The method generally employed for separation involves a precipitation of the inorganic P either as a molybdenum salt, or as the ammonium magnesium salt, but the latter technique is generally preferred because of the high absorbing power of molybdenum for β-radiation (Hevesy, 1948). Because of the rather great sensitivity of the methods employed for the measurement of the radioactivity, careful attention to the conditions of precipitation is necessary. Prime among the requirements is that ample time should be allowed for completeness of precipitation and that sufficient carrier P be present. Generally the ammoniacal solution, from which inorganic P is being precipitated, is kept overnight at 0–5° to ensure complete precipitation.

There is some doubt about the amount of carrier P which should be added. The literature shows that most workers add carrier P, but it is rare to find any reference to the amount which has, in fact, been added. Theoretically, since it is a question of measuring only ^{32}P, the amount of non-radioactive P which is present is of no consequence, provided that sufficient has been added to ensure complete precipitation of ^{32}P, and that correction factors are applied should the precipitate being counted exceed a limiting thickness. From the practical viewpoint, however, the amount of carrier P which is present is of considerable importance and it is the purpose of this communication to show that serious errors are introduced into the determination of the S.A. by the use of conventional techniques. A preliminary report of this work has already been published (Ennor & Rosenberg, 1951). A method will also be described, by means of which the accurate and rapid determination of the S.A. of inorganic P may be made in presence of labile organophosphates.

A recent publication by Ennor & Stocken (1950) has given an account of a method for the determination of inorganic P in the presence of acid-molybdate-labile organophosphates. Briefly, this method, which is a modification of that described by Berenblum & Chain (1938), involves the combination of molybdic acid and inorganic P in an acid aqueous phase and the extraction of the resultant phosphomolybdic acid with *iso*butanol. Both reaction and extraction are accomplished in 10 sec. and thus only the P released in this time interval from any acid-molybdate-labile organophosphates present adds to the inorganic P. It was shown that the P so measured is subject to an error equal to only 1% of the amount of P present as phosphocreatine, this being the most labile P compound present in most tissue extracts. It occurred to us that this method would be of value in experiments involving ^{32}P, since the

* In this paper the term 'inorganic phosphate' refers to that form of phosphorus which can be precipitated as magnesium ammonium phosphate and which will react directly, in acid solution, with ammonium molybdate to form a reducible phosphomolybdate.

use of a liquid counter and a colorimeter would permit the determination of both the radioactivity and P on the one solution.

It should be pointed out that Rowles & Stocken (1950) have used a somewhat similar technique, except that the original method of Berenblum & Chain (1938) was followed. In general, however, the procedure described by Ennor & Stocken (1950) is to be preferred because of the presence of phosphocreatine in extracts of most tissues and in particular of liver (Barker, Ennor & Harcourt, 1951).

The subject-matter of this paper was presented at the Brisbane Meeting of the A.N.Z.A.A.S. in May 1951.

METHODS

Two adult male guinea pigs were used for the experiments. Both were starved for 24 hr. prior to sacrifice, and in the case of one animal 50μc. of ^{32}P was injected intraperitoneally 4 hr. before death. The ^{32}P was in the form of NaH_2PO_4 and was injected in a volume of 3·0 ml. to which sufficient non-radioactive NaH_2PO_4 had been added to render the solution isotonic. The other animal was untreated.

Preparation of liver extracts

The animals were stunned by a blow on the head and the cervical vessels severed. The abdomen was quickly opened and the liver removed and dropped into liquid air. The frozen liver was weighed and then crushed in a mortar previously chilled to the temperature of liquid air. It was then transferred to a Nalco-type homogenizer, roughly weighed and homogenized for 2 min. in 10% (w/v) trichloroacetic acid (TCA). The suspension was then centrifuged and the supernatant poured off. A further extraction was carried out on the residue and, after centrifugation, the supernatants were combined and filtered to remove small particles. Sufficient 5N-NaOH was then added to the extract to adjust the pH to 7·3. During and after these operations, the temperature of the extract was maintained at 0–5°. After neutralization the volume of the extracts was so adjusted that the equivalent of 1 g. of liver was contained in about 18 ml.

Precipitation and preparation of precipitates of magnesium ammonium phosphate

Samples (5·0 ml.) of all experimental solutions and TCA liver extracts investigated were carefully pipetted into 15 ml. tubes. Magnesia mixture (1·0 ml.) (Vogel, 1947) was added, together with carrier P in the form of NaH_2PO_4 in amounts as detailed later and then sufficient water to bring the volume to about 10 ml. 4N-NH_4OH was then added dropwise until the solution was alkaline to phenolphthalein. At this stage, 0·6 ml. of NH_4OH (sp.gr. 0·88) was added. Stirring was effected by means of an air stream from a fine capillary, care being taken to avoid scratching the walls, in order to obviate difficulty in quantitative removal of the precipitate later. The tubes were then placed in the refrigerator (1°) and left for 18–24 hr. At the end of this time the precipitates were quantitatively transferred to a circle (diam. = 2·5 cm.) of Whatman no. 50 filter paper, mounted in a stainless steel modified Büchner type funnel, as described by Oddie & Lynn (1951). The supernatant liquid was drawn through by suction and the precipitate was washed several times with 0·5N-NH_4OH and finally with ammoniacal ethanol. The filter pad was then removed and mounted on a brass disk and fixed in position by an annular brass ring. The precipitate was finally dried under an infra-red lamp before counting.

^{32}P and ^{31}P determinations

(1) *Magnesium ammonium phosphate precipitates.* ^{32}P was determined under standardized geometry using a conventional decade scaler, power pack and GM4 end-window counter. All samples were prepared and counted in quadruplicate and for such times as resulted in reduction of the statistical error to $\pm 1\%$.

For the determination of P two techniques were adopted, depending upon whether the precipitate was ashed or not. In those experiments demanding ashing, the entire filter-paper disk was carefully removed from the brass mount and transferred to a micro-Kjeldahl tube. 10N-H_2SO_4 (1·0 ml.) and conc. HNO_3 (1·0 ml.) were then added and the precipitate and paper wet-ashed on an open electric furnace. After all traces of HNO_3 were removed by continued heating, about 5 ml. of water were added and the tubes immersed in boiling water for 20 min. to hydrolyse any pyrophosphate formed. The contents were then quantitatively transferred to a measuring flask and made up to 50 ml. in the case of those precipitates containing 0·1 and 0·5 mg. of carrier. The precipitates containing 1·0 mg. of carrier P were made up to 100 ml. Duplicate 5·0 ml. samples were pipetted into 50 ml. separating funnels containing 2·0 ml. of 5% (w/v) NaCl. Other reagents were added in the volumes, concentrations, and by the technique described by Ennor & Stocken (1950). The higher concentrations described by these authors were used throughout and the 10 sec. shake technique employed. As it separated, the aqueous layer was run off into a 25 ml. measuring flask and made up to volume by the addition of water. The alcohol layer was then washed by vigorous shaking for 10 sec. with two successive volumes, each of 10 ml. of N-H_2SO_4. Each wash (wash 1 and wash 2) was drawn off as in the case of the aqueous layer and made up to a similar volume. Reduction was carried out by shaking for 30 sec. with 15 ml. of $SnCl_2$ (Berenblum & Chain, 1938) in flasks reserved for that purpose and the $SnCl_2$ layer, as before, was made up to 25 ml. by the addition of water. The alcohol phase was quantitatively washed into a 25 ml. flask with ethanol and made to volume with this solvent. Optical density of the alcohol phase was determined as described by Ennor & Stocken (1950). Radioactivity of the alcohol and all the aqueous phases was measured in a liquid counter of 10 ml. vol. and of the type described by Veall (1948).

In the case of those precipitates which were not ashed, the filter-paper disk was transferred to a volumetric flask of appropriate volume and the precipitate dissolved by the addition of 2 ml. of 0·1N-HCl. The solution was then adjusted to volume by the addition of water. Samples (5·0 ml.) were taken and treated as in the case of those precipitates which had been ashed.

(2) *TCA extract.* Usually for the determination of both ^{32}P and P in the inorganic P a 1·0 ml. sample was sufficient and the procedure followed was as described above for the ashed precipitates, except that the aqueous layer and the two washes were retained only in the case of the second model experiment. In the subsequent experiment the aqueous layer contained labelled organophosphates, and

these were of no interest in this experiment. After reduction, the alcohol phase was retained for optical density and radioactivity determinations.

Expression of results

Some of the experiments quoted here were carried out over 48–60 hr. and in such cases the observed counts have been corrected for decay to the time at which the first count was made. In all cases, appropriate corrections have been made to the counts as measured in *iso*butanol and water, and these corrected results have been expressed as what they would have been had they been counted as $MgNH_4PO_4$ using the end-window counter.

RESULTS

^{32}P recoveries in model systems. The initial experiment was designed to determine the amount of inactive sodium dihydrogen phosphate necessary to ensure complete precipitation of the ^{32}P from an aqueous solution as the magnesium ammonium salt. Three different amounts of carrier were added, namely, 0·1, 0·5 and 1·0 mg. of P as sodium dihydrogen phosphate. From Table 1 it may be seen that a tenfold increase in the amount of carrier has produced only about a 5% increase in the radioactivity of the precipitate, from which it may be inferred that precipitation is practically complete at the 1·0 mg. level. Additional data on the completeness of precipitation is given below.

Table 1. *The influence of varying amounts of inactive carrier* P *upon the amount of* ^{32}P *precipitated from an aqueous solution*

P added as inactive NaH_2PO_4 (mg.)	$MgNH_4PO_4$ ppt. counts/min.
0·1	930
0·5	950
1·0	980

In the second model experiment a TCA extract was prepared from the liver of a normal untreated guinea pig and radioactive phosphate was added after neutralization. This extract thus contained unlabelled organophosphates and inorganic P, some of which was labelled. The results (Table 2) indicate that, since no radioactivity could be detected in the aqueous layer from which the inorganic P had been extracted as phosphomolybdate, the *iso*butanol must quantitatively extract the ^{32}P. In addition, it is also evident that the partition coefficient for phosphomolybdate in *iso*butanol and water is so overwhelmingly in favour of the alcohol phase that no losses of radioactivity occur in the two acid washes and in the stannous chloride subsequent to extraction.

In the experiments carried out with magnesium ammonium phosphate precipitates prepared as a result of the addition of 0·1 and 1·0 mg. of P as carrier, a result similar to that shown in Table 1 is seen; i.e. a tenfold increase in the amount of carrier produces only a 3% increase in the radioactivity. When *iso*butanol extraction was carried out on the precipitates in the manner described above, the radioactivity was recovered in the alcohol phase and no counts in excess of background could be detected in the various aqueous phases. The

Table 2. *Recovery of ^{32}P from TCA extract of normal guinea pig liver*

(^{32}P was added to this extract after preparation. No labelled organophosphates were present. All counts are the means of duplicate determinations and in no case did the variation between duplicates exceed 3%.)

	Counts/min.	
*iso*Butanol extract of TCA	1070	
Aqueous layer, Wash 1, Wash 2, $SnCl_2$ layer	0	
	*a**	*b*†
$MgNH_4PO_4$ ppt.	990	1020
*iso*Butanol extract of ppt.	1030	1050
Aqueous layer, Wash 1, Wash 2, $SnCl_2$ layer	0	0

* In presence of 0·1 mg. P as NaH_2PO_4 as carrier.
† In presence of 1·0 mg. P as NaH_2PO_4 as carrier.

counts in the *iso*butanol and in the precipitates should, of course, be equal in both '*a*' and '*b*' respectively and the differences noted may be ascribed to experimental error. In view of the 3% increase in counts which is obtained when the amount of carrier P increases from 0·1 to 1·0 mg. it is of interest to note that even at the higher level the counts in the precipitate are still about 5% less than those recorded in the *iso*butanol extract of the TCA extract. Since this latter must be presumed to be correct (there is no radioactivity in the various aqueous phases) it may be inferred that the ^{32}P is only about 95% precipitated in the presence of 1·0 mg. of carrier P.

On the basis of the foregoing experiments it may be concluded that the *iso*butanol extraction of inorganic P as phosphomolybdate can be expected to give accurate results for the determination of the radioactivity and thus the specific activity of inorganic P both in the absence and presence of unlabelled organophosphates. When, however, the method was applied to TCA extracts of livers from animals injected with ^{32}P, the radioactivity, as measured in the *iso*butanol extract, was invariably less than that measured on the magnesium ammonium phosphate precipitates. In one case, the

counts/min./ml. of TCA extract, as measured on the precipitate, were almost double those determined on the *iso*butanol (Ennor & Rosenberg, 1951). On the basis of the foregoing experiments, the *iso*butanol extract cannot be expected to give a low result, but a high result is possible if it is assumed that some of the labelled organophosphates may go into simple solution in this solvent—this was considered unlikely. The position is very different in the case of magnesium ammonium phosphate, since here a low or a high result is possible by virtue of incomplete precipitation on the one hand and co-precipitation and/or adsorption on the other. The difference then between the magnesium ammonium phosphate count and the *iso*butanol count was regarded as significant and indicative of the presence of some radioactive contaminant.

In order to determine the source of error an experiment essentially similar in nature to the last described was carried out. In this case the TCA extract was prepared from the liver of an animal injected with ^{32}P and thus this extract contained, in addition to labelled inorganic P, labelled organophosphates.

Table 3. *Comparison of results obtained in determination of radioactivity (counts/min.) of inorganic* P *by precipitation as* $MgNH_4PO_4$ *and by extraction of phosphomolybdate in* isobutanol

(TCA extract prepared from liver of guinea pig injected intraperitoneally with approx. 50 μc. ^{32}P as NaH_2PO_4 3·5 hr. before death. All results are expressed as counts/min./ml. of TCA extract.)

*iso*Butanol extract of TCA		1130	
	*a**	*b**	*c**
$MgNH_4PO_4$ precipitate	1270	1600	1740
*iso*Butanol extract of precipitate	1140	1110	1100
Aqueous layer	150	430	570
Wash 1, wash 2, $SnCl_2$	0	0	0
*iso*Butanol extract of ashed precipitate	1240	1540	1680
Aqueous layer	0	0	0
Wash 1, wash 2, $SnCl_2$	0	0	0
*iso*Butanol extract of Mg precipitate + aqueous layer	1290	1540	1670

* To *a*, *b* and *c* was added respectively 0·1, 0·5 and 1·0 mg. of non-radioactive P as carrier and the counts are the means of quadruplicate determinations.

On the basis of the results described above, the figure 1130 (Table 3) may be taken as representing the true value for the radioactivity of the inorganic P, and it will be noted that this figure is exceeded if the radioactivity is determined on the magnesium ammonium phosphate precipitate. Of much greater significance, however, is the fact that the radioactivity in the precipitate increases as the amount of non-radioactive carrier increases. This increase in radioactivity can only be explained by the assumption that co-precipitation of some labelled organophosphate(s) has occurred. Proof of this assumption is given by determination of the radioactivity in the *iso*butanol extract of the precipitate. This technique, which was described above under the heading 'Magnesium ammonium phosphate precipitates' will extract only that P present as inorganic P, and any organophosphates present will remain in that fraction referred to as the 'aqueous layer'. Thus the radioactivity of the *iso*butanol extract of the magnesium ammonium phosphate should be equal to that of the TCA extract. The results indicate that this is the case (Table 3), and it will be noted that the counts are, within the limits of experimental error, identical for each of the three precipitates. On the other hand, the counts in the aqueous layer increase as the amount of carrier P increases and the sum of such counts, plus those in the corresponding *iso*butanol extract, are approximately equal to the counts in the respective precipitates.

Ashing of the precipitates and subsequent treatment of a sample with molybdate and extraction with *iso*butanol should yield an *iso*butanol extract which contains the same number of counts as did the original precipitate when counted by the end-window counter. In addition, since all the P in the precipitate is now in the form of inorganic P, no radioactivity should appear in the 'aqueous layer'. Both contentions are supported by the results and it must, therefore, be concluded that the magnesium ammonium phosphate precipitates are contaminated by some ^{32}P-containing organophosphorus compound(s).

It has been noted by Lehninger (1949) that some co-precipitation of adenosinetriphosphate (ATP) occurs when inorganic P is precipitated as magnesium ammonium phosphate and it therefore seemed likely that some, at least, of the excess counts in the precipitate would be due to ATP. For the purposes of comparison, the absorption spectra of one of each of the three precipitates prepared as a result of the addition of 0·1, 0·5 and 1·0 mg. of carrier P were determined by means of the Hilger 'Uvispek' spectrophotometer. The absorption curves were identical with that of an authentic sample of ATP and all had an absorption maximum at 2570 A. In our experiments no attempt has been made to identify positively the contaminating compound(s) as ATP by specific enzymic analysis, and it has been assumed that the entire absorption at 2570 A. is due to ATP and the results are expressed as such in Table 4. It is appreciated, however, that the precipitate may contain, in addition to ATP, both the mono- and di-phosphates.

It will be noted that the amount of 'ATP' which is found in the precipitates increases as the mass of the precipitate increases and that with the addition

of 1·0 mg. of P as inert carrier the amount precipitated reaches 0·3 mg. It is of interest to compare this with the figure of 0·79 mg. which was the amount of inorganic P found to be present in 5·0 ml. of the TCA liver extract.

Table 4. *Relationship between the amount of carrier P added and 'ATP' precipitated*

(The extinction coefficient at 2570 A. has been assumed to indicate only the presence of ATP and the results are expressed accordingly. All results are expressed on the basis of a 5·0 ml. sample of the TCA extract of guinea pig liver and the precipitates referred to are those whose radioactivities are indicated in Table 3.)

	*a**	*b**	*c**
Weight of $MgNH_4PO_4$ ppt. (mg.)	1·25	5·2	10·6
Weight of ATP (mg.)	0·056	0·206	0·300
Excess counts/min.†	700	2350	3050
Excess counts/min. / μg. ATP	12·5	11·4	10·2

* As in Table 3 columns *a*, *b* and *c* refer respectively to the precipitates containing 0·1, 0·5 and 1·0 mg. of carrier P.

† Excess counts/min. = counts/min. in $MgNH_4PO_4$ ppt. − counts/min. in *iso*butanol extract of the TCA liver extract.

Calculation of the contribution which is made to the total radioactivity of the precipitate by this 'ATP' shows a close parallelism to the weight of 'ATP'. The ratio, excess counts/min. to μg. 'ATP' indicates this more clearly and it is evident that, if only one ^{32}P-containing compound was co-precipitated and if this was 'ATP', this ratio should be a constant. The ratio is, however, not a constant and it may thus be inferred that all three of the phosphorylated adenosine compounds are precipitated to varying extents as the amount of carrier P increases, or that possibly some other ^{32}P-containing compound(s) is also precipitated. This inference gains support from the fact that the ratio, excess counts/min. to μg. 'ATP' (both are calculated on the difference which results when the amount of carrier P increases from 0·5 to 1·0 mg., i.e. *c*–*b*) falls to 7·4.

Table 5. *Specific activity* (S.A.) *of inorganic phosphate*

$$\text{S.A.} = \frac{\text{counts/min. TCA extract}}{\mu\text{g. P/ml. TCA extract}}. \quad \text{Inorganic P} = 15{\cdot}9\ \mu\text{g./ml.}$$

Method of determination of radioactivity		Counts/min. TCA extract	S.A.
On *iso*butanol extract of TCA extract		1130	71
On the $MgNH_4PO_4$ precipitate	+0·1 mg. carrier P	1270	81
	+0·5 mg. carrier P	1600	100
	+1·0 mg. carrier P	1740	110

Finally, it is of interest to calculate the specific activities of the inorganic P fraction of the TCA liver extract on the basis of the observed content of inorganic P = 15·9 μg.P/ml.

The S.A. which is regarded as the true value is that determined by means of the *iso*butanol-extraction technique. As indicated from the foregoing experiments, the value obtained for the S.A. as determined on the magnesium ammonium phosphate precipitates by solid-counting technique depends on the amount of non-radioactive carrier P which is added and in the case of the largest amount of carrier P which we have employed (1·0 mg.) the positive error introduced amounts to over 50 %.

DISCUSSION

In view of the fact that precipitation of inorganic P as magnesium ammonium phosphate is generally accepted as the most desirable method for the determination of the radioactivity and hence the specific activity of the inorganic P of tissue extracts, it was expected that some investigation would have been made of the specificity of the precipitation. To the best of our knowledge no such investigation has been carried out and indeed, apart from two occasions, no doubts appear to have been cast on the accuracy of the S.A. so determined. Kalckar, Dehlinger & Mehler (1944) qualified the interpretations of their data with the remark 'provided that the ammonium magnesium precipitate represents only inorganic P...'. Kamen & Spiegelman (1948), in discussing techniques for differentiating between 'ortho' and 'organic' P fractions, pointed out that the extent to which magnesia mixture will precipitate only 'ortho' P has still to be assessed. The experiments described in this communication show conclusively that the precipitate represents considerably more than inorganic P and that as a result the calculated S.A. is subject to considerable error. Since, however, the magnitude of this error is a function of the amount of inert carrier P which has been added, it is clearly impossible to assess the error which has been introduced into the results of any particular worker who has worked with ^{32}P, unless the amount of carrier P added is known. It would also seem certain that the true turnover rate of any particular organophosphorus compound must be greater than that quoted. This follows from the fact that the true S.A. of the inorganic P is less than that actually measured by conventional methods and that the S.A. of any particular organophosphate must, therefore, be a larger fraction of the true S.A. of the inorganic P than of the S.A. measured. The extent to which this argument is valid will depend on the compound, the methods used for its separation and on the composition of the extract. Thus, in the case of phosphocreatine, if the ^{32}P is freed by mild hydrolysis and then precipitated as the magnesium ammonium salt, the error introduced would conceivably be 'matched' to some extent by an error of similar magnitude in the case

of inorganic P. It is assumed, of course, that both hydrolyses and precipitations are carried out in unfractionated tissue extracts, but the argument may still hold if carried out on partially fractionated extracts. In this paper no attempt has been made to assess the error introduced with amounts of carrier in excess of 1·0 mg. P and our results indicate that precipitation of ^{32}P is incomplete at this level. Since other workers have claimed complete precipitation it may be assumed that the amount of carrier P added is greater than 1·0 mg. and hence that the results so obtained suffer from an error greater than 50%. In view of the doubts which arise as a result of the present work it would seem worthwhile to re-investigate the turnover rates of those phosphorus-containing compounds whose precursors are believed to be inorganic P.

When this work was completed the work of Sacks (1951) came to our notice. He has stated that adenosine triphosphate-adenosinediphosphate is adsorbed on the magnesium precipitate and that it amounts to 20% of the total amount present. This statement, in the light of the present results, is ambiguous, since the amount of 'ATP' precipitated depends upon the bulk of the precipitate. The amount of ATP precipitated (as can be calculated from our results) can approach 100% of that present. In this connexion it is interesting to recall that Sacks (1940) was unable to precipitate ATP from an alkaline filtrate after precipitation of the inorganic P as magnesium ammonium phosphate. This failure may be explained by the present results and would seem due to the fact that all of the ATP was in the precipitate and not in the filtrate.

The procedure adopted by Sacks (1951) in dissolving the magnesium ammonium phosphate in acid, and precipitating the P as a molybdate, may also be expected to give erroneous results by virtue of the hydrolysis of the adenosinetriphosphate-adenosinediphosphate when heated in 2N-nitric acid at 60°.

The *iso*butanol technique detailed in the present paper is free from all the errors which characterize the conventional precipitation techniques, and has the added advantage that it is rapid and permits the determination of the radioactivity and the amount of P present in the one solution. The method has the additional advantage that it can be used for the determination of both P and ^{32}P released from any organophosphate by enzymic or chemical hydrolysis.

Addendum. A recent publication by Ernster, Zetterström & Lindberg (1950) has been brought to our notice. These workers have described a method for the determination of the specific activity of inorganic phosphate in tissue extracts which in principle is the same as that described here. Certain disadvantages are, however, inherent in the Martin & Doty (1949) modification of the Berenblum & Chain (1938) technique which was used. Thus the aqueous phase is in contact with the *iso*butanol-benzene extractant for 1 min. during which time hydrolysis of labile phosphorus compounds will occur. Also the practice of adding $SnCl_2$ in the presence of ethanol to form a single phase system with the *iso*butanol-benzene is not to be recommended, because of the extensive etching of glass cells and liquid counters which occurs. Ernster *et al.* (1950) have shown that their method, like ours, gives a lower result for the specific activity of inorganic phosphate than does the Mg precipitation technique. On the other hand, it does not seem to have been appreciated that the error is, in part at least, due to the presence of adenosine polyphosphates and that the magnitude of the error depends upon the amount of carrier P which has been added.

SUMMARY

1. The accepted technique of precipitating the inorganic phosphorus present in tissue extracts as magnesium ammonium phosphate for the determination of the radioactivity has been investigated and shown to be subject to considerable error.

2. The magnitude of this error depends on the bulk of the precipitate and is due to co-precipitation and/or adsorption of organophosphates among which are the adenosine polyphosphates.

3. The errors involved in determining the specific activity have been discussed, and it is concluded that the turnover rates of organophosphates as previously determined are probably lower than the true values.

4. A method has been described which permits the accurate and rapid determination of both the radioactivity and the amount of phosphorus present in the one solution.

It is a pleasure to express our thanks to Dr D. M. P. Phillips of this Department for determination of the absorption spectra and to Miss J. D'Alton for technical assistance. Our thanks are also due to Dr T. H. Oddie of the Radio Tracer Section of C.S.I.R.O. for advice on the preparation of samples of $MgNH_4PO_4$ for determination of radioactivity and for much assistance in the installation of the counting apparatus. It is also a pleasure to express our thanks to Dr F. G. Morgan, Director of the Commonwealth Serum Laboratories and to the Director General of Health, for a generous provision of laboratory space pending the establishment of this Department in Canberra.

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Some Effects of Administering Ergothioneine to Rats

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The presence of ergothioneine in erythrocytes is well established. This has been made possible by the delicate and highly specific Hunter diazo reaction (Hunter, 1928, 1949). Latner (1948) used a modified diazo reaction to determine ergothioneine in blood ultrafiltrates, while Lawson, Morley & Woolf (1950) precipitated ergothioneine with iodobismuthous acid prior to colorimetric determination by the Hunter reaction. Touster (1951) has published a method for the estimation of ergothioneine in which the thiol group is oxidized with bromine water to sulphate, which is precipitated as benzidine sulphate and determined colorimetrically. The reaction of certain mercaptoglyoxalines with 2:6-dichloroquinone-chloroimide described by McAllister (1951*a*, *b*) might also be applicable to the determination of ergothioneine, but like Touster's method would not have the specificity of the Hunter reaction.

Ergothioneine has been stated to occur in human, rat, pig, ox, cat, guinea pig, dog, fowl and pigeon erythrocytes in amounts up to 30 mg./100 ml. of whole blood. There is no report in the literature of any species in which ergothioneine is entirely absent. It is found exclusively in the red cells and is not present in the plasma (cf. Fraser, 1950). Variations of ergothioneine blood levels with geographical location have been recorded by Hunter (1951) for men, rats and pigs. Significant increases in diabetic patients have been reported by Salt (1931) and by Fraser (1950). Latner & Mowbray (1948) observed lower ergothioneine levels in some thyrotoxic patients and raised levels in some cases of myxoedema.

Ergothioneine is the only 2-mercaptoglyoxaline known to occur naturally, and has been found only in blood, in ergot and in some other fungi. See, however, Leone & Mann, 1951. The possibility that erythrocyte ergothioneine is derived from dietary ergothioneine seems to be remote, and the report of Eagles & Vars (1928) that a maize diet increased the blood ergothioneine level in pigs has not been confirmed by Hunter (1951). Hunter also showed that when ergothioneine or 2-thiolhistidine was given to young rats (5 mg./animal) for 3 days, there was no increase in blood ergothioneine 2 days later. As will be shown later, however, the erythrocyte concentration of ergothioneine can be greatly increased by feeding rats a diet containing 0·1 % ergothioneine, although the increase is gradual.

The question of the occurrence of ergothioneine in urine has been examined by various workers. Sullivan & Hess (1933) and Work (1949) claimed that it was excreted in appreciable amounts, whilst Woolf (1949) and Lawson *et al.* (1950) failed to detect any by paper chromatography. The last named workers also reported that a single injection of ergothioneine in the rat did not bring about any detectable excretion during the following 24 hr. The use of a new procedure which has likewise given no indication of the presence of ergothioneine in

normal urine has, however, established that a considerable proportion of ergothioneine administered to rats either subcutaneously or in the diet is rapidly excreted.

The synthesis of ergothioneine by Heath, Lawson & Rimington (1950, 1951) made possible the preparation of sufficient quantities for feeding experiments, the main objects of which were to determine the fate of ergothioneine after administration, and to show conclusively whether or not ergothioneine has any antithyroid activity.

Lawson & Rimington (1947) reported that administering ergothioneine to rats caused hyperplasia and decreased the total iodine content of the thyroid glands. No such activity was found, however, by Astwood & Stanley (1947), Wilson & McGinty (1949) or by Searle, Lawson & Hemmings (1950), although Pitt-Rivers (1948) found that ergothioneine, like the known antithyroid drugs, inhibited the *in vitro* conversion of acetyldiiodotyrosine into acetylthyroxine. Since even a slight antithyroid activity might have significance in a substance naturally occurring in blood, ergothioneine has now been administered continuously to rats at a higher level than previously reported and in a diet of very low iodine content. Under these conditions even a weak antithyroid activity would be expected to cause appreciable changes in the weight, iodine content and radioactive-iodine uptake of the thyroid gland. No such activity has, in fact, been found.

MATERIALS AND METHODS

Rats. Hooded rats of the Glaxo strain were used in groups of six to seven animals, matched for weight and sex. They were weighed daily, commencing about 4 days before adding ergothioneine to the diet.

Diet. Glaxo powder diet RBSS-9 was used throughout. No iodine was detected in this diet when 0·25 g. samples were analysed as described below. Ergothioneine was incorporated in the diet at a level of 0·1 %, calculated as free base.

Ergothioneine. Synthetic ergothioneine was prepared by the method of Heath *et al.* (1951). It was not resolved. Natural ergothioneine hydrochloride was converted to the free base by dissolving in the minimum amount of water, and adjusting to pH 7 with NH_3; ergothioneine rapidly crystallized on adding excess methanol.

Iodobismuthous acid. This was prepared and used as described by Lawson *et al.* (1950). After storage for 3 months in the dark at 4° the reagent contains appreciable amounts of free I_2 and its use leads to incomplete precipitation of ergothioneine and bad colour development in the Hunter reaction. Extraction with $CHCl_3$ after dilution with an equal volume of water restored its original properties.

Hunter diazo reagent. Prepared and used as described by Hunter (1949).

Blood ergothioneine. Blood samples were obtained by heart puncture on the ether-anaesthetized animals immediately before killing. Ergothioneine was then determined as described by Lawson *et al.* (1950).

Urinary ergothioneine. Urinary ergothioneine was detected by the use of ion-exchange resins. A quantity of the total urine output collected over the 14-day period of feeding ergothioneine was diluted ten times with water and passed through a column of Zeo-Karb 215 (The Permutit Co., London, W. 4) which was then washed with water till the washings no longer gave the Folin-Marenzi reaction. The column was fractionally eluted with CO_2-free 0·1 N-NH_3, those fractions giving a positive Folin-Marenzi reaction being bulked and subsequently adsorbed on a Dowex 2 (Dow Chemical Co., Michigan, U.S.A.) column. After washing, the ergothioneine was obtained by fractional elution with CO_2-free 0·1 N-HCl and was free from all impurities interfering with the Hunter test. Slight but variable ergothioneine losses occurred on the Zeo-Karb, but not on the Dowex columns, and for the determination of ergothioneine in urine the more rapid method described below was preferred.

Determination in urine. A tenfold diluted urine (1 ml.) from rats receiving ergothionine was heated on the boiling-water bath for 3 min. with 1 ml. water and 1 ml. 5 % (w/v) sulphosalicylic acid solution in 0·03 N-HCl. After cooling and centrifuging, a sample (2 ml.) of the clear liquid was treated with $HBiI_4$ and the diazo reaction carried out on the precipitate as described by Lawson *et al.* (1950). Addition of choline as co-precipitant was unnecessary. The colour intensities were determined with a Pulfrich photometer using filter no. S53. For the blank determination the same procedure was followed using normal rat urine, and a calibration curve was made by substituting 1 ml. of an aqueous solution containing known amounts of ergothioneine for the water.

Determination of ergothioneine in rat tissues. Samples of the liver, kidney, spleen, adrenals and thyroids representative of the group of rats were homogenized with 2·3 % (w/v) aqueous sulphosalicylic acid and diluted with this reagent to give a 10 % extract. After centrifuging, the ergothioneine in 2 ml. of the supernatant liquid was determined after precipitation with $HBiI_4$ as described above. Separate calibration curves were made for liver, kidney and spleen, using samples of the corresponding tissues from normal animals. This was justifiable since no ergothioneine could be detected in liver homogenates from normal rats using the chromatographic procedure described above for urine.

Radioactive iodine. Radioactive iodine (^{131}I) was obtained from Atomic Energy Research Establishment, Harwell. 1 μc., free of added carrier, was injected intraperitoneally in 0·5 ml. of 0·9 % NaCl solution into each animal 24 hr. before it was killed with $CHCl_3$. The uptakes of ^{131}I by the thyroids were then determined as described by Searle *et al.* (1950).

Iodine analyses. Materials for analysis were ashed with $KMnO_4$-H_2SO_4, and the iodine was distilled with oxalic acid (Talbot, Butler, Saltzmann & Rodriguez, 1944), and determined colorimetrically by the As_2O_3-$Ce(SO_4)_2$ procedure (Taurog & Chaikoff, 1946). Natural and synthetic ergothioneine (25 mg. samples) and the normal and supplemented diets (0·25 g. samples) have been analysed for iodine in this way.

RESULTS AND DISCUSSION

Blood ergothioneine

The ergothioneine blood level of all the rats used was less than 1 mg./100 ml. whole blood at the start of these experiments. The level slowly increased

Table 1. *Effect of* 0·1 % *ergothioneine in the diet of rats, for varying periods, on thyroid weight and* ^{131}I *uptake and on ergothioneine blood levels*

(The diet supplemented with synthetic ergothioneine contained additional I (0·6 μg./g.). No ergothioneine was found in the blood of control rats.)

Ergothioneine	Time fed (days)	Change in thyroid weight (%)	Change in ^{131}I uptake of thyroids (%)	Blood ergothioneine of dosed rats (mg./100 ml.)
Natural	2	−13·3	+0·8	<1
Synthetic	2	−14·2	−41·8*	<1
Synthetic	4	+0·8	−35·6*	2·0±0·1
Natural	11	−14·6	−16·8	7·2±0·8
Synthetic	11	−19·5*	−68·9*	6·15±0·7
Synthetic	14	−2·1	−45·5*	10·2±1·0

* Statistically significant ($P < 0.05$).

when the diet was supplemented with either natural or synthetic ergothioneine, reaching 2 mg./100 ml. after 4 days and 10 mg./100 ml. after 14 days (Table 1). No ergothioneine was found in the plasma. The slow rate of incorporation of ergothioneine into the blood does not conflict with the preliminary observation of Hunter (1951) that a single dose of ergothioneine given orally for 3 days does not increase the blood level.

An experiment was also performed with oxalated human blood, incubated at 37°, to acertain whether or not ergothioneine could enter the erythrocytes from the plasma. Portions of blood (2 ml.) were used for a preliminary determination of the ergothioneine levels in plasma and cells, then to the remaining 10 ml. of blood was added 1 mg. of ergothioneine dissolved in 0·1 ml. of normal saline and the mixture was incubated with gentle agitation. Samples (2 ml.) were withdrawn for analysis immediately after the addition and at hourly intervals up to 4 hr. The cells were spun down, washed three times with normal saline, and plasma and cells analysed for ergothioneine. No transference of ergothioneine from plasma to cells could be detected. This finding, together with the gradual increase in erythrocyte ergothioneine which occurred during the feeding experiments, would suggest that ergothioneine normally present in blood is incorporated during erythropoiesis.

Urinary ergothioneine

There was no detectable difference in the feeding experiments between the behaviour of natural and synthetic ergothioneine. In both cases some ergothioneine was excreted in the urine within 24 hr. after a single subcutaneous injection (Table 2), although at this time no detectable quantity was present in either the erythrocytes or the plasma, suggesting that ergothioneine has a very low renal threshold in the rat.

Tissue ergothioneine

The ergothioneine found in the spleen (Table 2) after 14 days on the ergothioneine diet could be due to the blood content of this organ, but this is not the case with the liver and kidney, which undoubtedly have the power of accumulating ergothioneine. In another series of experiments, to be reported later, it was found that the ergothioneine content of rat liver 48 hr. after discontinuing the administration of the substance was 60 mg./100 g. liver and levels as high as 100 mg./100 g. have been reached in certain cases. No ergothioneine could be detected in the adrenals or thyroid gland, and no differences in the ergothioneine distribution among the different tissues were observed with different routes of administration of the ergothioneine or between natural and synthetic ergothioneine.

Table 2. *Distribution of ergothioneine* 24 *hr. after a single subcutaneous injection of* 15 *mg. synthetic ergothioneine to a* 150 *g. rat and at the end of a* 14*-day period on a diet containing* 0·1 % *synthetic ergothioneine*

	Ergothioneine concentration (mg./100 g.*)		
	Control diet	After single subcutaneous injection	After 14 days 0·1 % dietary ergothioneine
Urine	<1	74	83
Blood	<1	2·8	10
Kidney	<1	21	40
Liver	<1	27·5	36·5
Spleen	<1	—	5
Adrenals	<1	—	<1
Thyroids	<1	—	<1

* Except for urine and blood, where concentrations are in mg./100 ml.

Effect on thyroid activity

Synthetic and natural ergothioneine have been administered as described for periods up to 23 days. In no experiment has any change in growth rate,

or any significant increase in thyroid weight indicative of antithyroid activity been observed. The results are shown in Table 1.

After 23 days on the synthetic ergothioneine diet, the effects observed were only those attributable to the presence of a small amount of iodine (0·6 μg./g. of diet) present as an impurity in the ergothioneine, namely, a significantly decreased thyroid weight (19·9 % below control weight) and a significantly greater thyroid iodine concentration (41·5 % above control values). Under these conditions even a weak antithyroid activity would have reversed such effects. As a result of this slightly increased iodine content of the synthetic ergothioneine diet, the ^{131}I-uptake method gave a false impression of activity when this diet was fed owing to dilution of the ^{131}I with non-radioactive iodide. Such dilution effects are an ever-present potential source of misleading results when diets of low iodine content facilitating the detection of weak antithyroid activity are used. With the diet supplemented with natural ergothioneine no significant depression of the 24 hr. uptake of ^{131}I was found after 2 or 11 days.

There seems little doubt, therefore, that the antithyroid activity of ergothioneine is negligible.

SUMMARY

1. Methods for the determination of ergothioneine in tissues and urine are described.

2. Contrary to previous reports it is found that when ergothioneine is administered to rats, either orally or subcutaneously, some is rapidly excreted in the urine.

3. Ergothioneine administered in the diet is rapidly accumulated in the liver and more slowly in the erythrocytes. No ergothioneine has been detected in the plasma at any time during these experiments.

4. Ergothioneine does not exert any antithyroid action in the rat even when tested under conditions which would reveal any slight activity.

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The Histochemical Demonstration of a Specific Phosphatase (5-Nucleotidase)

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Animal tissues contain a specific phosphatase named 5-nucleotidase, which attacks only nucleotides with the phosphate group attached to C-5 of a pentose (Reis, 1937, 1940, 1951; Heppel & Hilmoe, 1951). The object of the present study was to adapt Gomori's method for histochemical demonstration of alkaline phosphatase (Gomori, 1939, 1946), to the demonstration of 5-nucleotidase.

Gomori (1949) and Newman, Feigin, Wolf & Kabat (1950) investigated the specificity of phosphatases using Gomori's histochemical method. This consists of the incubation of tissue sections fixed in cold acetone in a buffered solution containing a phosphoric ester and a calcium salt. The orthophosphate produced by enzymic hydrolysis forms insoluble calcium phosphate, which is precipitated on the slide and visualized by one of the methods adopted for staining calcium in the tissues. Many of the results of the above authors differed greatly from those which we have obtained by chemical estimations. Newman *et al.* (1950) failed to find any histochemical evidence of 5-nucleotidase activity in thyroid and testicle, although extracts of both these tissues have a high 5-nucleotidase activity (Reis, 1951), and Gomori was unable to demonstrate 5-nucleotidase in adrenal gland or placenta, both of which were found by Reis (1937) to contain this enzyme in moderate amounts. These differences may be explained partially by the inactivating action of the histological fixation, but it seems probable also that the pH (9·2) used by Gomori and by Newman *et al.* was not suitable for demonstrating 5-nucleotidase activity, as this enzyme has its optimum near pH 8·0.

Gomori, and Newman *et al.* estimated the presence of 5-nucleotidase by comparing two slides incubated at pH 9·2, one with adenylic acid, the other with β-glycerophosphate. The difference between the two was taken as corresponding to the activity of 5-nucleotidase, since adenylic acid is hydrolysed by both 5-nucleotidase and by alkaline phosphatase, whereas β-glycerophosphate is hydrolysed by the alkaline phosphatase only. Such results may be clear enough when 5-nucleotidase is present in the examined tissue in the absence of alkaline phosphatase. If both enzymes are present, however, it is difficult to judge whether the heavier precipitates with adenylic acid are really due to 5-nucleotidase action. Not only is the degree of precipitation not exactly proportional to enzyme activity, but the visual comparison is also subject to error.

The different activity of alkaline phosphatase against various phosphoric esters has also to be taken into account. Taking, for an example, the phosphatase activities of an extract from rabbit lung (Fig. 1), at pH 9·0 it is obviously difficult to say whether the difference between the adenylic acid and β-glycerophosphate hydrolysis is due to the 5-nucleotidase activity, or whether it is due to a different activity of alkaline phosphatase on adenylic acid. This varying activity of alkaline phosphatase is illustrated by the hydrolysis of phenylphosphate which, as was shown by King & Delory (1939), at pH 9·0 is about four times as fast as that of β-glycerophosphate, while that of adenylic acid is between the two.

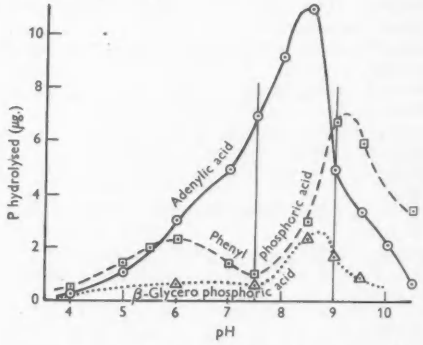

Fig. 1. Enzymic hydrolysis of phosphoric esters by an extract of rabbit lung. 5 mg. of tissue (dialysed extract), 20 min., temp. 38°, substrate concentration 0·0008 M. Details of technique as described by Reis (1951).

The chemical estimation of 5-nucleotidase activity was carried out at pH 7·5, since this is near to the optimal pH of this enzyme (pH 7·8 in human tissues), and also because at this pH the rates of hydrolysis of phenylphosphate and β-glycerophosphate are both very low at 7·5, and the hydrolysis of adenylic acid by non-specific phosphatases has a

similar low value. In subtracting such a small value from the total hydrolysis of adenylic acid, we probably make a much smaller error than in subtracting the far larger values for non-specific hydrolysis obtained at pH 9·2. It therefore seemed worth while to try this pH for the histochemical method also, in spite of the increase in the solubility of calcium phosphate and the consequent expectation of an increase in diffusion of this product. The additional advantage of estimations at pH 7·5 is that they give a picture of enzyme activities at the physiological pH.

METHOD

Thin blocks of tissue were fixed, according to Gomori's (1946) method, in two or three changes of cold absolute acetone at 4° for 24 hr. They were then transferred progressively to absolute ethanol, absolute ethanol-ether with one or two changes, and thence to 1% celloidin. After draining off any excess of the latter the blocks were hardened in $CHCl_3$, cleared in benzene and embedded in paraffin wax. Sections were cut at 5 μ. and mounted on albuminized slides. Heating on the hot stage was avoided by drying the slides for 3 hr. in a 37° incubator. Slides and blocks were then stored at 4° until required for incubation. This latter process was carried out by removing the wax from the slides by immersion in light petroleum and smearing a line of vaseline round the sections to retain the buffer-calcium-substrate solutions. When these had been added the slides were put into Petri dishes or into Perspex boxes, with a few drops of distilled water nearby to minimize evaporation. They were incubated at 37° for varying times. The necessary development of calcium phosphate precipitates required from 3 to 24 hr., according to the degree of enzyme activity.

To demonstrate the 5-nucleotidase activity three slides were incubated, as a rule, at pH 7·5, one as a blank test with water instead of substrate, one with phenylphosphate and one with adenylic acid. The difference between the sections incubated with adenylic acid and phenylphosphate corresponds to the action of 5-nucleotidase. Generally, at pH 7·5, phenylphosphate incubation gave no Ca precipitate. To demonstrate alkaline phosphatase activity an extra slide was usually incubated with phenylphosphate at pH 9.

Solutions

Buffer for 5-nucleotidase (pH 7·5). Sodium diethylbarbiturate, 0·1 M, 3 vol.; HCl, 0·1 N, 2 vol.; distilled water, 1 vol.

Buffer for alkaline phosphatase (pH about 9). Sodium diethylbarbiturate 0·1 M, 1 vol.; distilled water, 1 vol.

Substrates. Sodium phenylphosphate, sodium β-glycerophosphate, or adenylic acid, 0·04 M.

The pH values of the substrates and the Ca and Mg solutions were adjusted to 7·5 (with 0·1 N-HCl or NaOH).

The incubating fluids were prepared by mixing 5 vol. of a buffer solution with 1 vol. of 12% (w/v) $Ca(NO_3)_2$, 1 vol. of 2% (w/v) $MgCl_2$ and 1 vol. of substrate solutions.

After incubation the slides were washed with 2% $Ca(NO_3)_2$ of pH about 8·0, covered with 1% $AgNO_3$ and exposed to daylight for about 1 hr. The Ag solution was then removed with distilled water and the slides covered with 5% $Na_2S_2O_3$ for 10 min. They were then washed, dehydrated in ethanol, cleared in xylene and mounted in neutral Canada balsam or in a synthetic medium such as D.P.X. (Kirkpatrick & Lendrum, 1941). The latter causes rapid fading of the precipitates if the cobalt method is used, but only slight deterioration of Ag precipitates.

The $AgNO_3$ method of visualization of calcium phosphate was preferred to the $Co(NO_3)_2$-yellow $(NH_4)_2S$ method, since it gave darker pictures and less non-specific staining.

RESULTS

Fig. 2 shows the action of alkaline phosphatase in rabbit lung at pH 9·0 (substrate: phenylphosphate, 3 hr. incubation). The sites of maximal alkaline phosphatase activity are the bronchial epithelium and the endothelia of arteries and veins. The staining of the nuclei may be an active enzymic process, but is almost certainly due to the diffusion of calcium phosphate. The control slides incubated without substrate and with phenylphosphate or β-glycerophosphate at pH 7·5, showed after 3 hr. no precipitate in either case. Fig. 3 illustrates adenylic acid hydrolysis at pH 7·5 (3 hr.) in a serial section to that of Fig. 2. The precipitate corresponds entirely to 5-nucleotidase activity. Here the medial coat of the artery shows the most intense staining, and only some adjacent nuclei are also stained. Further away from the maximum sites of activity the nuclei are unstained, indicating that this effect is due to diffusion. When adenylic acid was used at pH 9, both medial coat and endothelia were stained.

In tissues where chemical estimations indicated substantial 5-nucleotidase activity this was confirmed by the histochemical method. In human thyroid, aorta or nerve we obtained pronounced staining after 18 hr. incubation with adenylic acid at pH 7·5, whereas these tissues showed only traces of alkaline phosphatase activity, when phenylphosphate or β-glycerophosphate at pH 9 were used for the same period.

Figs. 4 and 5 show the alkaline phosphatase and 5-nucleotidase activity of the human pituitary gland. The substantial 5-nucleotidase activity of the posterior pituitary was first demonstrated histochemically, and afterwards confirmed by chemical estimations (Reis, 1951). Incubation for 3 hr. was quite sufficient to get a good staining with this tissue; in no other human tissue was a comparable 5-nucleotidase activity found.

DISCUSSION

The use of adenylic acid at pH 7·5 allows the histochemical demonstration of 5-nucleotidase activity, which at this physiological pH is in many tissues much greater than that of the non-specific phosphatases. The staining method which we have evolved is therefore interesting in that it reflects

physiological conditions, but it suffers from the disadvantage that the solubility of calcium phosphate is much greater at pH 7·5 than at 9, so that diffusion phenomena are greater. It is therefore impossible, with the method described, to localize 5-nucleotidase within a single cell; only the activity of a group of cells or a whole tissue may be demonstrated. Finer differences in calcium phosphate deposition are due to diffusion.

Gomori (1950) makes the objection that the interpretation of phosphatase activity by his method, when performed between pH 7·4 and 8·4, is very doubtful, especially when long incubation times are used (more than 48 hr.). Our incubations never exceeded 24 hr., and in the case of rabbit lung and human pituitary gland they lasted 3 hr. only. In view of the diffusion of calcium phosphate at pH 7·5, with the 5-nucleotidase method incubation should be restricted to periods of 6 hr. or less and not prolonged to 24 hr. or more, as in the case of alkaline phosphatase. In Fig. 3 the effect of diffusion may be evaluated by the staining of the nuclei, and it can be observed to affect a limited area only. The histochemical findings confirm the chemical estimations, in other tissues as well as in artery wall and posterior pituitary.

We are well aware that by the method described only the sites of maximal 5-nucleotidase activity are demonstrated. The sites of lower 5-nucleotidase activity, as well as the sites of alkaline phosphatase activity, are usually not visualized at pH 7·5, but the positive reaction with adenylic acid at pH 7·5 cannot be considered as an artifact since it corresponds to the chemical estimations and is to a large extent proportional to the time.

SUMMARY

1. A method has been evolved, based on Gomori's alkaline phosphatase method, for histochemical demonstration of specific phosphatase (5-nucleotidase) activity, by incubation with adenosine-5′-phosphate at pH 7·5.

2. Examples of 5-nucleotidase staining are given, and the limits of localization by this method are discussed.

3. In human tissues, the strongest 5-nucleotidase staining was found in the posterior pituitary gland.

We should like to thank Mr J. G. Griffin and Mr L. Wright for technical assistance in the preparation of the sections and Mr E. V. Willmott for the photomicrographs.

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EXPLANATION OF PLATE 4

Fig. 2. Rabbit lung. Stained for alkaline phosphatase (phenylphosphate, pH 9) which is present (top) in the endothelium of a large artery, and (bottom) in the epithelial lining of a bronchus. (×100.)

Fig. 3. Rabbit lung (serial section to Fig. 2). Stained for 5-nucleotidase (adenylic acid pH 7·5) which is concentrated in the medial coat of the artery and absent from the endothelium. (×100.)

Fig. 4. Human pituitary gland. Posterior part at the top, anterior below. Stained for alkaline phosphatase (phenylphosphate, pH 9). (×18.)

Fig. 5. Human pituitary gland (serial section to Fig. 4). Stained for 5-nucleotidase (adenylic acid, pH 7·5). (×18.)

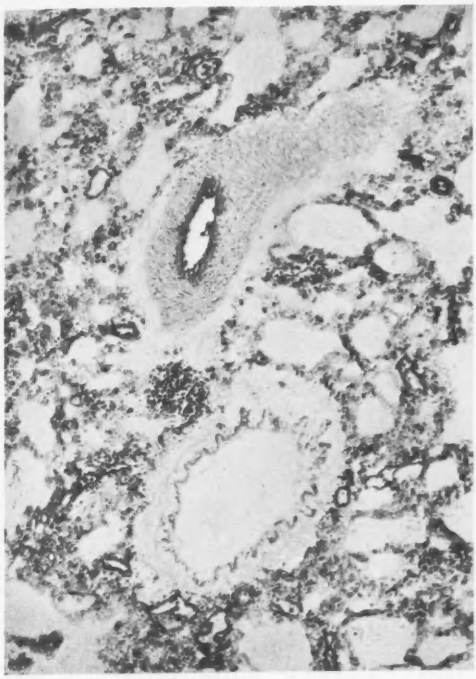

Fig. 2.

Fig. 3.

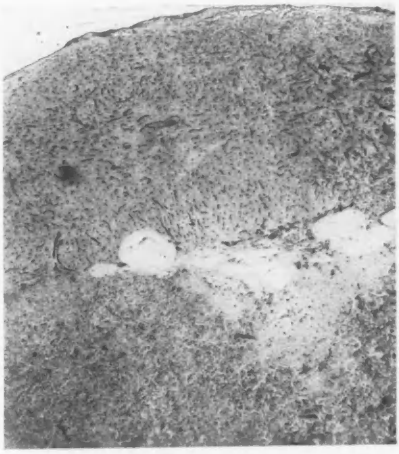

Fig. 4.

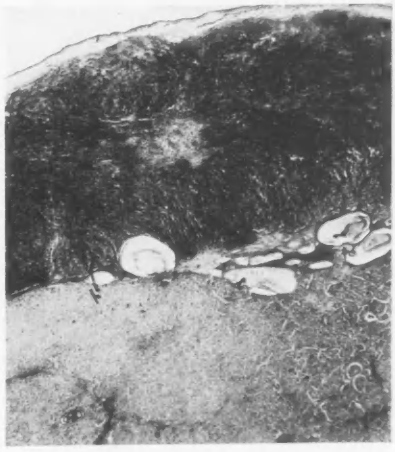

Fig. 5.

A. G. E. PEARSE AND J. L. REIS—THE HISTOCHEMICAL DEMONSTRATION OF A SPECIFIC PHOSPHATASE (5-NUCLEOTIDASE)

Studies on the Skeletal Tissues

2. THE COLLAGEN CONTENT OF BONES FROM RABBITS, OXEN AND HUMANS

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It has been recognized for many years that collagen forms an important constituent of the organic matrix both of bones and of the dentine of teeth, but precise analytical information has been scanty. Lightfoot & Coolidge (1948) examined the collagen content of the tissues of the guinea pig and concluded that 68–70% of the total nitrogen in the whole skeleton was collagen. No examination was made of individual bones. Other results, however (Weidmann & Rogers, 1950), have shown that, even when thoroughly cleaned, there is a difference in the calcium to nitrogen ratio between cancellous and cortical bone taken from the femora of small laboratory animals, and variations in the nitrogen and ash content have been shown even in different parts of the shafts of long bones from rabbits (Dallemagne, 1943) and from oxen (Strobino & Farr, 1949). Changes also occur in the ash content of the skeleton of animals with increasing age (Hammett, 1925; Hess, Berliner & Weinstock, 1931; Strobino & Farr, 1949), and in the phosphorus to nitrogen ratio (Roche & Garcia, 1936); Strobino & Farr (1949) quote unpublished results in support of an increase with age in the ash content of human bone. It is difficult to interpret these differences and changes in the absence of more precise knowledge of the nature of the matrix, since the total nitrogen content of bone indicates only the amount of total protein present and the value obtained may be influenced by occluded plasma or by a layer of periosteum not removed during cleaning. When ash content is measured these uncertainties become even greater since the presence of non-nitrogenous organic substances and carbon dioxide can contribute to the result obtained for the amount of matrix. It is thus impossible to be certain that variations in either calcium to nitrogen ratio or in ash content correspond to degrees of calcification of a single substance.

In the present work a study has been made of the 'collagen' content of different types of bone, with various calcium to nitrogen ratios, obtained from rabbits and oxen and also of femora from human subjects of different ages. It is believed that the results obtained indicate a variability in the degree of calcification of a matrix which consists predominantly of a single protein, present in all types of bone at all ages.

METHODS

Rabbit bones from healthy adult animals were dissected immediately after death. Oxen femora were obtained freshly from the slaughter house. The human samples were of mid-femur taken at autopsies performed on children and adults dead as the result of accident or of such acute illnesses as were thought, by a competent pathologist, not to affect the skeleton. The cleaning, drying and defatting of all the samples was carried out as previously described (Weidmann & Rogers, 1950). Instead of grinding the bones with a dental drill as before, they were crushed between steel surfaces, a modification necessary to eliminate heat production which leads to the formation of considerable amounts of water-soluble nitrogen (Stack, 1950*a*).

The collagen content of the crushed, dried and defatted samples was estimated as follows. The powders were decalcified with N-HCl (15 ml./g. powder) for 18 hr. at 0–4°. The insoluble materials were removed by centrifugation, washed three times with water, each time adjusting the pH of the suspensions to 7·0, resuspended in 10 ml. of water and autoclaved for 2 hr. at 20 lb. pressure. The suspensions were then centrifuged whilst still hot, and the precipitates washed thrice with 10 ml. amounts of boiling distilled water. The original supernatants and the washings were cooled to 15° and made up to 50 ml. or to a suitable fraction of this volume if less than 1 g. of crushed bone had been available in the first instance. Samples (2·5 ml.) were taken from each of these solutions into centrifuge tubes, the pH adjusted to 4·6 with N-HCl, using bromocresol green as indicator, and 5·0 ml. amounts of 5·0% (w/v) tannic acid added. The mixtures were cooled for 1 hr. at 0–4°, centrifuged, and the precipitates dissolved in 5·0 ml. of 2 N-NaOH by breaking them up with a glass rod and warming (3–5 min.) in a boiling-water bath. Total nitrogen estimations (two from each sample) were made on the solutions by the micro-Kjeldahl technique using copper selenite and potassium sulphate as oxidation catalysts in the preliminary incineration which was continued for 18 hr. This method for collagen estimation is a necessary modification of that used by Lightfoot & Coolidge (1948) since, both in our experience and in that of Stack (1950*b*), attempts to extract gelatin from whole bone powder with the technique used by these authors lead to very low recoveries of nitrogen owing to adsorption of the proteins on the insoluble inorganic salts.

Total nitrogen estimations were also made on the samples of the whole bone powder, the decalcifying fluid and on the material not rendered soluble by autoclaving. Two estimations were made for each sample, and the results throughout this paper are given as the mean of these duplicates. The overall recovery of nitrogen for several of the samples recorded in this paper is slightly greater than 100%. It is presumed that this is partly due to the sampling error involved in determinations of total nitrogen in the bone powders and partly to the normal distribution curve of the experimental error. The average recovery of nitrogen throughout all the experiments performed is 99%.

Phenolic compounds, presumably tyrosine, in the material rendered soluble by autoclaving were estimated by use of the Folin & Ciocalteu phenol reagent. A preliminary treatment of the solutions for 15 min. with 0·5 N-NaOH (equal volumes of the solutions and N-NaOH were mixed) at 100° was found to be necessary in order to prevent the precipitation which otherwise occurred when the phenol reagent was added.

RESULTS

Collagen content of rabbit bones

The results expressed in Table 1 show that from 89 to 97% of the total nitrogen in all the rabbit bones examined is present as collagen, or exists in such a form in the decalcified matrix that it can be removed by autoclaving at neutral pH and subsequently precipitated by tannic acid. There is certainly no suggestion that there is less collagen in bones with a relatively high nitrogen content. For example, the cancellous bones of the femurs examined contain 4·5 and 4·1% nitrogen, whereas the shafts from this bone contain only 3·0 and 2·8%, yet the proportion of collagen in the cancellous structures is higher than that in the shaft. The differences are small and a large group of animals would have to be examined to verify any general statement, but it appears that there is a tendency for there to be a slightly higher proportion of collagen in the cancellous than in the shaft bone.

A further indication of this tendency is given by the amount of nitrogen from the matrix which is not brought into solution by autoclaving. The cancellous tissues from the long bones contain only 0·6–1·1%, the hard shaft bone 2–8%, of the nitrogen in this form. A preliminary examination (Rogers & Hall, 1951) of the amino-acid composition of the hydrolysed insoluble material, which will be reported at greater length in a later publication, suggests that it may contain a high proportion of elastin. If this is so, then the presence of larger amounts in the shaft bone is understandable, since the walls of the numerous blood vessels will contribute elastin, whereas all except the largest trabeculae are probably free of vessels. The material rendered soluble by autoclaving shows an amino-acid pattern similar to that for skin collagen (Bowes & Kenten, 1949).

There are no regular differences in the small amounts of nitrogen which are dissolved from different types of bone during decalcification. Fractionation and examination of this latter material shows that it contains some collagen and some nitrogen volatile when the solution is made alkaline. The latter is presumed to be ammonia. It may be noted that vigorous washing with water will remove an amount of total nitrogen similar to that removed by the acid during decalcification.

An examination of the amount of phenolic substances present in the crude gelatin solutions was made because it is known that collagen prepared in a highly purified state from various sources has a low content of aromatic amino-acids. Neumann (1949), for example, quotes values of 2·3–2·6% for phenylalanine, 0·86–1·1% for tyrosine and <0·01% for tryptophan in samples of collagen prepared from eleven different sources. Moreover, a low content of substances absorbing ultraviolet light at a wavelength of 2575–2875 A. has been suggested (Loofbourow, Gould & Sizer, 1949) as a criterion for the purity of collagen. The figures in column 10 of Table 1 are the amounts of phenolic substances (expressed as μg. tyrosine/mg. total nitrogen) present in the solution of the materials removed

Table 1. *Collagen content of samples of rabbit bones*

(The results recorded under I are for bones from a single animal, whilst those under II are for powders prepared as pools from bones of three animals.)

	mg. N/g. dry defatted bone powder								Recovery of N (%)		Tyrosine (μg./ml.) / Total N (mg./ml.)
	Total*		Soluble†		Collagen		Insoluble‡				
Type of bone	I	II	I	II	I	II	I	II	I	II	II
Femur shaft	30·2	28·3	1·25	0·95	26·9	26·4	2·41	1·72	101	102	91·5
Femur cancellous	45·3	41·3	2·03	0·93	43·8	—	0·28	0·46	101	—	73·2
Tibia shaft	36·3	33·4	1·07	0·99	32·8	29·7	1·92	0·68	93	94	84·0
Tibia cancellous	47·2	42·9	—	1·16	—	42·0	—	0·28	—	101	74·2
Scapula compact	44·2	38·7	1·0	0·79	41·8	36·1	1·67	1·96	100	100	65·5
Scapula blade	50·9	45·6	0·84	0·82	49·2	—	0·35	0·44	99	—	77·1
Vertebrae cancellous	41·9	46·7	1·50	1·11	40·3	44·8	0·31	0·50	100	99	81·9

* The total N content of 1 g. of dry defatted bone powder in mg.
† Found in the decalcifying fluid.
‡ Not rendered soluble during autoclaving.

from the decalcified bone by autoclaving. It will be seen that there is a considerable degree of variation in the values obtained. Two explanations are possible: either the amino-acid composition of collagen may vary according to its source, or, more probably, small amounts of proteins, polypeptides or amino-acids other than collagen and its derivatives may be brought into solution when the matrix is autoclaved. It is of some interest that the solutions prepared from the cancellous bone contain less phenolic substances than those from shaft bone. Thus it seems likely that the matrix of the cancellous bones not only contains more material rendered soluble by autoclaving, but also less of the contaminating substances, containing a higher proportion of aromatic amino-acids, which can also be brought into solution during the process. This matter will be discussed at greater length below.

The collagen content of oxen bones

According to Dallemagne (1943) and to Strobino & Farr (1949) there is a maximum in ash and minimum in nitrogen content at a characteristic point in each shaft bone from rabbits and oxen. It seems therefore of importance to know the proportion of collagen in various parts of such bones. In order to do this disks of bone from ox femora were cut as indicated in Fig. 1*a*. Three samples were then chipped from each of the disks (Fig. 1*b*) except from that of the epiphyseal bone which was sampled from the centre of the disk and well away from its cartilagenous edge. Thus, altogether, thirteen samples were taken, each of which was crushed, defatted and analysed as previously described.

The results obtained from a typical experiment are shown in Table 2. It will be seen that, as Strobino & Farr (1949) reported, there is considerable variation in nitrogen content. The values for collagen are similar to those shown in Table 1 for the rabbit bones, with the exception that there is less evidence for a higher proportion of collagen in the samples with a high nitrogen content. In all the samples the collagen nitrogen makes up about 91–96 % of the total. There is again some fluctuation in the amount of material not rendered soluble by autoclaving, but the relationship between this and the total nitrogen in the bone is not so clear as it is in the rabbit skeleton. For example, despite the high nitrogen in the epiphyseal sample, the 'autoclave insoluble' material makes up about 1 % of the total as compared with 2–4 % for the samples of shaft bone with the lowest total nitrogen.

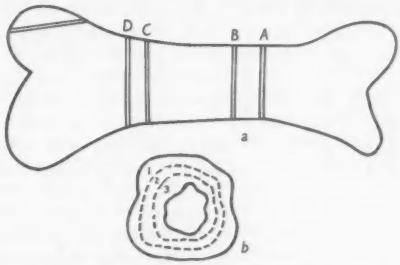

Fig. 1. Method of sampling ox femora.

Consideration of the amount of phenolic substances present in the gelatin solutions prepared from the oxen bones together with the results obtained using rabbit bones leads to an interesting conclusion graphically represented in Fig. 2. It will be seen that there is indication of an inverse correlation between the amount of total nitrogen and the proportion of phenolic substances. Thus the pro-

Table 2. *Collagen content of samples of bone from different parts of an oxen femur*

(See Fig. 1*a* and *b* for the position of the samples.)

Sample	mg. N/g. dry defatted bone powder				Recovery of N (%)	Tyrosine (μg./ml.) Total N (mg./ml.)
	Total*	Soluble†	Collagen	Insoluble‡		
A 1	36·9	0·88	33·4	1·57	97	69·7
A 2	36·2	0·67	33·0	1·54	97	73·2
A 3	38·2	0·73	36·4	0·98	100	83·8
B 1	35·8	0·88	34·2	1·49	102	82·9
B 2	35·1	0·71	33·7	0·78	100	76·2
B 3	37·3	0·97	34·8	0·69	98	86·8
C 1	35·8	0·98	33·9	0·70	99	70·0
C 2	39·1	0·91	35·8	1·54	98	55·7
C 3	42·2	0·86	39·5	1·10	98	53·2
D 1	38·5	0·99	35·8	0·78	99	58·1
D 2	40·2	0·88	38·6	0·90	100	59·6
D 3	41·9	1·06	40·3	0·96	100	69·7
Epiphysis	46·2	0·85	44·2	0·64	99	85·7

* Total N content of 1 g. of dry defatted bone powder in mg.
† Found in the decalcifying fluid.
‡ Not rendered soluble by autoclaving.

visional conclusions drawn from the results for the rabbit skeleton are confirmed and it seems probable that in these two species of animal the higher the nitrogen content of the bone the lower will be the degree of contamination of the collagen matrix with small amounts of other proteins, relatively rich in aromatic amino-acids.

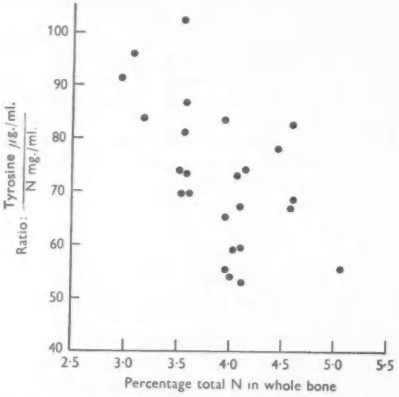

Fig. 2. Scatter diagram showing the relationship between total N in the bone and the proportion of phenolic substances in the autoclave-soluble material.

Changes in the calcium to nitrogen ratio and the collagen content of samples of human femur

Preliminary results (Rogers, 1949), with a few samples, suggested that there was an increase in the calcium to nitrogen ratio with the age of the subject from which femur samples had been taken. In the present series some forty samples have been taken from subjects ranging in age from 9 months to 90 years, and in Fig. 3 the results for calcium to nitrogen ratios are plotted against age. It will be seen that there is a steep rise in this ratio up to an age of about 20–30 years which corresponds to the time of closure of the epiphyses. Later in life there may still be a very slow rise, though the spread of the figures is too great to allow any definite conclusion to be drawn. If, however, the figures are plotted semi-logarithmically they fall on an approximately straight line which suggests that the increase is continuous throughout life. No evidence was found for a decalcification of the bone in advanced age, and thus the reduction in radiographic density which is sometimes referred to as decalcification is probably due largely to a reduction in the amount of bone and not to any change in its degree of calcification. These results agree with the statement made by Strobino & Farr (1949) and are paralleled by their results for oxen bones. They are, however, in conflict with Vogt's (1949) findings. The latter author examined

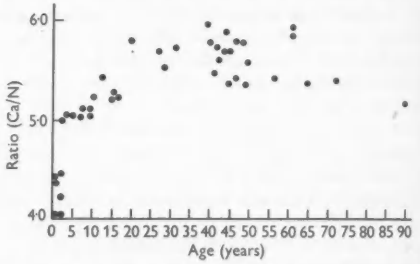

Fig. 3. Scatter diagram showing the relationship between Ca/N ratio and the age of the human subject from which the bone was taken.

the ash content of samples of iliac crest taken by biopsy from normal subjects of from 2 days to 73 years of age. He could detect no significant differences between the samples. It may be that not all the bones in the skeleton alter in their content of ash with age. These latter results would nevertheless be more convincing if some other constituent, such as total nitrogen which varies inversely with the ash content, had been measured at the same time, since one parameter alone is liable to be influenced by a number of factors such as the completeness of drying and cleaning of the samples.

A selection of the samples of human femur has been examined for collagen content by the usual technique. Table 3 shows the results obtained; as

Table 3. *Collagen content of samples of human femur taken from subjects of various ages*

Age of subject (years)	Sex	mg. N/g. dry defatted bone powder				Recovery of N (%)	Tyrosine (μg./ml.) / Total N (mg./ml.)
		Total*	Soluble†	Collagen	Insoluble‡		
2·5	M.	48·3	1·01	46·4	2·02	102	65·6
7	M.	49·7	0·94	47·7	0·42	99	69·0
10	M.	49·3	1·03	44·2	1·99	96	69·6
15	M.	47·8	0·68	44·3	1·38	97	72·6
27	M.	47·3	0·87	45·2	0·68	99	73·3
41	F.	46·5	0·95	43·6	1·49	99	75·9
65	M.	46·7	1·02	41·3	3·11	97	70·2

* Total N content of 1 g. of dry defatted bone powder in mg.
† Found in the decalcifying fluid.
‡ Not rendered soluble during autoclaving.

with the bones from the other species 89–96 % of the total nitrogen behaves as collagen. There is wide fluctuation in the values for the small amounts of material not rendered soluble by autoclaving, but there is no correlation with the age of the subjects from which the samples were obtained. No evidence can be found for the presence of a lower proportion of collagen in the bones from younger subjects having a lower ratio of calcium to nitrogen, and there is no trend in the reverse direction as there is with the animal bones. There is also no significant variation in the concentration of phenolic substances in the autoclave extracts; with one exception, the bone from the infant 2·5 years of age, the ratios of tyrosine in μg.:total N in mg. agree with the average value for the series to within $\pm 2{\cdot}1$ %. Thus there is no evidence for the presence of more contaminating protein of any kind in the collagen matrix from bones of young subjects than in that from the older ones.

DISCUSSION

Such a survey as this might clearly be extended almost indefinitely through the skeletons of any number of species. It is thought, however, that sufficient has been done to justify the statement that the organic matrix of bones from skeletons of mammals including *Homo sapiens* consists almost exclusively of a protein which is rendered soluble by autoclaving at neutral reaction and is subsequently precipitable by tannic acid. Such a protein can be considered to be a collagen, and this supposition is supported by preliminary examinations of its amino-acid content. Variations in the ratio of calcium to nitrogen, whether due to type of bone or to age of subject, can be safely regarded as differences in degree of calcification of this collagen. Thus the differences observed by Dallemagne (1943), Strobino & Farr (1949) and Weidmann & Rogers (1950) can be said to reflect differences in degree of calcification. Hitherto the criticisms made by Baker, Butterworth & Langley (1946) of claims for such variations were difficult to refute. The cleaning of intricate structures such as the epiphysis and metaphysis is difficult and the inclusion of minute amounts of extraneous protein would be sufficient to cause a marked alteration in the calcium to nitrogen ratio. The latter authors' own results for cancellous and cortical human bone gave point to these criticisms since they found that if the cleaning were adequate then the calcium to nitrogen values for different types of bone showed no statistically significant difference.

The question naturally arises as to how far collagens from the different bones are identical both with one another and with this type of protein isolated from other sites in the body such as the skin. We have obtained no results which will give guidance in this matter since it seems unlikely that the variations in the concentration of phenolic substances in the crude autoclaved extracts from the bone matrix indicate differences in the amino-acid composition of the collagens. It seems more likely that they are due to lack of specificity in the technique for collagen estimation and to consequent inclusion of small amounts of other proteins rich in aromatic amino-acids together with the collagen. As far as the relation between skeletal collagen and that from other sites in the body is concerned, Neumann's (1949) thorough examination of this protein from a variety of sources including bone shows that, although there are small variations, these are of dubious significance and that the amino-acid composition is probably constant.

Two sets of correlations have been observed in examining the results for animal bones. First, the higher the total nitrogen in the bone the lower is the content of phenolic substances in the autoclave extracts. Secondly the higher the proportion of collagen in the bone the lower are the amounts of substances not rendered soluble by autoclaving. Both these relationships can be adequately explained by the presence of a greater number of blood vessels in some samples than in others. The walls of the vessels might be expected to contribute proteins not rendered soluble by autoclaving. The plasma in the vessels will contribute polypeptides and proteins containing higher concentrations of aromatic amino-acids. This hypothesis is consistent with the presence of larger numbers of vessels in the shaft bone than in the epiphysis, and is supported by the larger content of phenolic substances and of 'autoclave-insoluble' nitrogen present in the former type of bone from rabbits.

SUMMARY

1. The organic matrix in the bones of rabbits, oxen and humans consists of from 90 to 96 % collagen, or collagen-like proteins.

2. Variations in the amount of total nitrogen relative to calcium in bones correspond to true differences in degree of calcification, and not to the inclusion of proteins other than collagen.

3. The degree of calcification of mid-femur samples of human bone increases with increasing age of the subject. This process is rapid up to the age of 20–25 years, but probably continues much more slowly throughout life.

4. The crude gelatin extracts from those rabbit and oxen bones which have a low calcium to nitrogen ratio are less contaminated by small amounts of other proteins than are those from the more highly calcified structures. This may possibly be correlated with the presence of blood vessels in the latter.

We take pleasure in acknowledging the help given by Dr D. Collins who selected the human material for us, by the Medical Research Council who provided one of us (S.M.W.) with a grant, and by the Nuffield Research Organization which gave the original grant of money which initiated the unit.

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A Search for Specific Chemical Methods for Fission of Peptide Bonds

1. THE *N*-ACYL TO *O*-ACYL TRANSFORMATION IN THE DEGRADATION OF SILK FIBROIN

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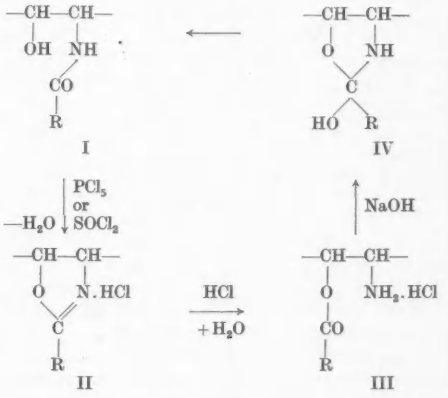
Partial degradation of proteins or polypeptides by chemical or enzymic hydrolysis is lacking in specificity and results obtained in this way have generally been very complex and difficult to interpret. Interest in the chemistry of the natural amino-acids has quickened considerably in recent years, the more complex amino-acids receiving particular attention. In the light of this recent knowledge it seems possible that the polypeptide chain could be broken in a predictable fashion by using a reagent showing selective reactivity with one amino-acid. It would be expected that only those amino-acids possessing functional groups in addition to the α-amino and carboxyl groups could be specifically attacked. The results described below show that serine displays distinctive chemical reactivity of the required type.

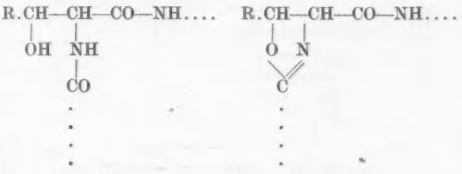
The migration of acyl groups in acyl derivatives of substances having a primary amino group and a hydroxyl group on adjacent carbon atoms was first observed in an aliphatic system by Bergmann, Brand & Dreyer (1921). Later, Bergmann & Miekeley (1924) demonstrated similar transformations in acyl derivatives of serine. The transformation of an *N*-acyl derivative (I) to an *O*-acyl derivative (III) and reconversion of the latter to the *N*-acyl derivative involved a number of steps which Bergmann, Brand & Weinmann (1923) showed to be as follows:



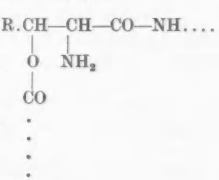
Compounds of types II and III were capable of independent existence and several examples were described by Bergmann and his collaborators, but compounds of type IV were assumed to have merely a transitory existence. Bergmann *et al.* (1923) were aware of the implications of their work in the protein field and put forward the suggestion that linkages such as those represented in II and IV might be found in proteins. Bergmann & Miekeley (1924) tried to substantiate this view by preparing an

oxazoline (type II) from glycylserine by treatment with thionyl chloride, but Bergmann, Miekeley & Kann (1925) showed that the compound obtained was 3-methylene-2:5-diketopiperazine.

In recent years a great many examples of the *O, N* migration have been discovered in compounds of varied type and have found important application in synthetic work, particularly where stereochemical problems were involved. Examples are to be found in the ephedrine field in which a careful study has been made by Welsh (1947) and in the synthesis of threonine (Elliott, 1948, 1949, 1950). As a result of methylation studies Blackburn, Middlebrook & Phillips (1942) suggested that oxazoline rings might be present in wool and silk. More recently Desnuelle & Casal (1948) have shown that in proteins the peptide bonds in which serine and threonine are linked through their nitrogen atoms are more labile than other peptide bonds to acid hydrolysis. This effect was more marked at low temperatures in the presence of very strong acid. These authors explained their results by assuming the intermediate formation of oxazoline rings in the peptide chain which were opened to give substances containing *O*-peptidyl bonds, the latter being more easily ruptured by acid hydrolysis than *N*-peptidyl bonds.



Normal peptide Oxazoline peptide



O-peptidyl form

Synge (1939) showed, however, that acid hydrolysis of DL-*N*-acetyl-*O*-benzoylserine yielded DL-*O*-benzoylserine, thus demonstrating the stabilizing effect of a charged nitrogen atom attached to a carbon atom adjacent to an ester group. On first consideration, the explanation offered by Desnuelle & Casal (1948) might appear to be incompatible with the observation of Synge (1939), but it should be pointed out that the yield of DL-*O*-benzoylserine obtained by Synge (1939) was small and it was not known whether any DL-*N*-acetylserine was produced simultaneously. A satisfactory comparison of relative rates of hydrolysis of *N*- and *O*-acyl bonds could only be made in a compound such as *ON*-diacetylserine in which both acyl groups are the same. The analytical data presented by Desnuelle & Casal supported the view that a shift of the peptide chain from nitrogen to oxygen in the linkages involving serine and threonine had occurred; the question whether or not an *O*-peptidyl bond is less stable than an *N*-peptidyl bond to acid hydrolysis cannot be decided on the evidence at present available. As a result of the work of Synge (1939) it seems probable that fission of an *O*-peptidyl bond by acid hydrolysis would require conditions at least vigorous enough to hydrolyse the more labile of the *N*-peptidyl bonds also. An attempt made by Desnuelle & Casal (1948) to bring about a rearrangement in egg albumin by the use of thionyl chloride was unsuccessful.

The purpose of the present investigation was to find a method of treating the protein molecule so that rearrangement of *N*-peptidyl linkages to *O*-peptidyl linkages occurred at the serine and threonine residues without at the same time causing hydrolysis of peptide bonds. It should then be possible to bring about a specific cleavage of the molecule. It seemed probable that the methods used by Bergmann and his co-workers in simple organic model substances would not be useful for proteins because these methods involved the use of organic solvents and vigorous reagents such as thionyl chloride and phosphorus pentachloride. In recent years ethanolic hydrogen chloride has been used for *N, O* acyl rearrangements (see, for example, Phillips & Baltzly, 1947), but it seemed unlikely that this reagent would be useful in the protein field. Reitz, Ferrel, Olcott & Fraenkel-Conrat (1946) found that when proteins were treated at low temperature with anhydrous sulphuric acid no peptide-bond fission took place unless the reaction was prolonged. The aliphatic hydroxyl groups were converted into mono-esters of sulphuric acid and some ring sulphonation occurred. Evidence supporting the view that no peptide-bond fission had occurred was obtained by Glendenning, Greensberg & Fraenkel-Conrat (1947) who found that insulin retained its biological activity after sulphuric acid treatment. Reitz *et al.* (1946) observed, however, that prolonged reaction of proteins with sulphuric acid caused a deep-seated change. This was indicated by the loss of sulphate bound by aliphatic hydroxyl groups, by the high amino-nitrogen values and by the low recoveries obtained when the crude reaction mixtures were poured on to crushed ice, neutralized and dialysed. This isolation procedure gave high recoveries with proteins given a short treatment with sulphuric acid. When the reaction with sulphuric acid was prolonged, breakdown of the molecule could be avoided by isolating the product in such a way as to avoid contact with

aqueous acid. These results could be interpreted on the assumption that the protein molecule had undergone rearrangement in a fashion analogous to the Bergmann transformations described above. As a result of the work of Reitz *et al.* (1946) it seemed probable that sulphuric acid would prove to be a very valuable reagent for the purpose in hand.

The experiments described below were concerned entirely with silk fibroin. This protein was chosen for study because of its availability, its relatively simple amino-acid composition and its high serine content. The degradation process consisted of three stages: (1) reaction of the protein with concentrated sulphuric acid at 21°, resulting in 'stage I protein'; (2) acetylation at pH 5, resulting in 'stage II protein'; (3) hydrolysis with dilute alkali, resulting in 'stage III acetylpeptide mixture'.

Rearrangement of silk fibroin with concentrated sulphuric acid

Treatment of silk fibroin with 97·5 % sulphuric acid at 21° for 3 days resulted in 62 % of the peptide bonds involving serine amino groups being converted into the *O*-peptidyl form. The extent of this conversion was not increased on further standing of the reaction mixture or under various other reaction conditions. It is shown in Table 1 that the amino

Table 1. *Amino* N *values found on stage I and stage II proteins compared with the amino* N *of soluble silk*

	Percentage of total N		
	Stage I protein	Silk fibroin	Stage II protein
Amino N	9·2	0·55*	0·53
Amino N after keeping at pH 9	2·0	—	—
Difference (amino N due to *O*-peptidyl bond formation)	7·2	—	—

* The value given for silk fibroin is that found on the soluble form prepared as described by Coleman & Howitt (1947).

nitrogen in the resulting stage I protein was considerably diminished after the material had been kept in a buffer solution at pH 9 for several hours. This behaviour would be expected from a substance having an *O*-peptidyl structure, because it would have been reconverted to the normal *N*-peptidyl form by the action of the alkali. It has been known for many years that acyloxyamines are extremely labile above pH 7; this transformation to *N*-acylaminoalcohol is generally instantaneous and complete. It is believed, therefore, that the difference between the two amino N values obtained before and after keeping stage I protein in alkaline buffer is a quantitative measure of the number of serine residues involved in the rearrangement. Rees (1946) found that the serine N in silk fibroin formed 11·6 % of the total N; on the basis of this figure the number of serine residues involved in the rearrangement was calculated to be 62 % of the total number present.

The results presented in Table 1 also show that the amino N of stage I protein after keeping in buffer solution at pH 9 was higher than the amino N of soluble silk. It is possible, therefore, that some non-specific fission of peptide bonds may have occurred in the reaction with sulphuric acid. The results obtained by the 1-fluoro-2:4-dinitrobenzene (FDNB) technique, on the other hand, indicate that only serine was involved in the reaction.

Experiments with fluorodinitrobenzene

It was not possible to react stage I protein under the usual conditions with FDNB because the reverse transformation from *O*-peptidyl to *N*-peptidyl form took place so rapidly, as is shown by comparison of Exp. 1 with Exp. 9 (Table 2). FDNB was found to

Table 2. *Amount of serine* N *in stage I protein which reacted with* FDNB *under various conditions of* pH, *time and temperature*

(Results expressed as a percentage of the serine N available for reaction: see Table 1.)

Exp. no.	Temp. (°)	pH	Time (hr.)	Serine N reacted (%)
1	20	5	4	23·4
2	20	5	8	26
3	20	5	19	19·2
4	20	5	19*	18·1
5	1·5	5	22	6·3
6	37	5	4·25	15·2
7	20	6	24	3·1
8	20	6	72	11·8
9	20	8·5†	2·5	4·5

* Experiment carried out in darkness.
† Using $NaHCO_3$ as buffer.

be sufficiently reactive to combine with amino groups at pH 5, at which the *O*-peptidyl form was stable. The 2:4-dinitrophenyl (DNP) derivative obtained was then hydrolysed under three different sets of conditions as described by Porter (1950), and the resulting DNP-amino-acids were separated on silicagel columns. The band due to DNP-serine was very much stronger than other bands; no band due to DNP-threonine was observed. The only other substance identified with certainty was DNP-glycine, the remaining bands being so weak that they were not considered to be significant. Glycine was also found as an end group in silk fibroin itself. The yield of DNP-serine obtained from DNP-stage I protein was much greater than the yield of any of the DNP-amino-acids obtained from DNP-silk fibroin, but in spite of many attempts (see Table 2) the number of DNP residues introduced into stage I protein was less than expected from the amino N results given in

Table 1. It does not follow that the presence of glycine as an end group in stage I protein was an indication of hydrolysis, because this amino-acid was also present as an end group in silk fibroin. Further experiments with FDNB showed that a number of other end groups besides glycine were present in silk fibroin; owing to this complexity and also to the uncertainty of achieving quantitative reaction of the FDNB with silk fibroin or with stage I protein, it was not considered practicable or useful to make a quantitative comparison of the results obtained on the two substances.

It was hoped that specific fission of DNP-stage I protein could be brought about by mild alkaline hydrolysis, yielding a mixture of DNP-seryl-peptides by fission of the *O*-peptidyl bonds. Some preliminary experiments on the partial hydrolysis of DNP-stage I protein were carried out. Mild alkaline hydrolysis was not successful, but partial acid hydrolysis gave promising results. These experiments were abandoned, however, when it was realized that there was very little hope of improving the reaction of FDNB with stage I protein.

The results of dialysis experiments presented in Table 3 show that some breakdown occurred when silk fibroin was treated with concentrated sulphuric acid. It is not known whether the loss of nitrogen was due to destruction of some specific part of the fibroin molecule or whether it was the result of general breakdown of a small fraction of the total number of molecules. This question could not have been decided without a full amino-acid analysis of stage I protein. The value found for serine N on the stage III acetylpeptide mixture and recorded in Table 6 is not very different from the value of 11·6 % of the total N found for serine N in silk fibroin by Rees (1946). It is possible, therefore, that the soluble non-diffusible material recovered from the reaction with sulphuric acid had not suffered any change of amino-acid composition. The insoluble material remaining after dialysis was not examined.

Table 3. *Indiffusible* N *present at each stage in the degradation of silk fibroin*

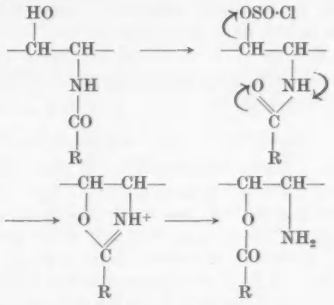
(Results expressed as a percentage of the total N in the system immediately before dialysis; for conditions see Experimental section.)

	Indiffusible N (%)
State I protein	65 (soluble) 22 (insoluble)
State II protein	97
Stage III acetylpeptide mixture	14

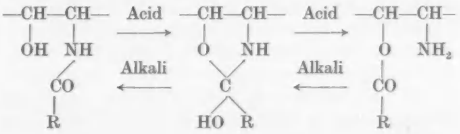
Gordon, Martin & Synge (1943) found that only about 50 % of the serine hydroxyl groups present in silk fibroin could be methylated by the action of dimethyl sulphate and alkali, in spite of continued treatment of the protein with the reagents. This finding could be correlated with the present observation that only 62 % of the bonds involving serine amino groups were rearranged by sulphuric acid; the reason for this lack of reactivity, however, is not understood.

Mechanism of the rearrangement

As a result of recent work it is evident that more than one mechanism can operate in the rearrangement (cf. Welsh, 1949; Phillips & Baltzly, 1947). Thionyl chloride forms oxazolines from acylamino-alcohols and to obtain the acyloxyamine it is necessary to treat the oxazoline with dilute acid. Elliott (1949) has shown that when the carbon atom carrying the hydroxyl group is asymmetric then an inversion of configuration may occur on that carbon atom on treatment of the substance with thionyl chloride:



The use of this reagent involves a two-stage process from acylaminoalcohol to acyloxyamine. Phillips & Baltzly (1947) have shown, however, that ethanolic hydrogen chloride causes rearrangement of acyl-aminoalcohols with production of relatively small amounts of oxazoline. These authors are probably correct in assuming that a hydroxyoxazolidine is the intermediate in this case, the oxazoline being formed by a side reaction involving dehydration of the hydroxyoxazolidine. In support of the conclusions of Phillips & Baltzly (1947) it has been found that DL-*N*-benzoyl*allo*threonine ethyl ester is converted into the corresponding *O*-benzoyl derivative without change of configuration by the action of ethanolic hydrogen chloride. The hydroxyoxazolidine structure, which was postulated many years ago by Bergmann *et al.* (1923) as an intermediate in the reverse transformation under the influence of alkali seems now to be well established as an intermediate when proceeding in either direction:



It cannot be decided at present which mechanism operates in the rearrangement of silk fibroin with concentrated sulphuric acid. In some of the preliminary experiments on the reaction of silk fibroin with sulphuric acid the protein was isolated in the dry state by precipitation and washing with dry ether. No evidence was obtained for the presence of oxazoline rings in this material: the amino N value remained unchanged after standing for several hours at pH 1, but the sulphuric acid used was not completely anhydrous and it is possible that oxazoline rings were produced and immediately opened again by the water present. It would be possible to establish the mechanism of the rearrangement using sulphuric acid, if threonine could be induced to take part in the reaction, by examining the configuration of the threonine obtained on acid hydrolysis of the stage I protein, but at present there is no evidence suggesting that threonine behaves in the same fashion as serine. In order to decide this question it would be better to use a protein containing a higher proportion of threonine than does silk fibroin.

Acetylation of stage I protein at pH 5

It has already been pointed out that a charged nitrogen atom attached to a carbon atom adjacent to an ester group renders this group more stable to acid hydrolysis than an ordinary ester group. It seemed probable, therefore, that it would not be very easy to find conditions for the preferential fission of the *O*-peptidyl linkages in stage I protein by acid hydrolysis, because of this stabilization phenomenon. Alkaline hydrolysis was impossible owing to the intervention of the reverse transformation from *O*-peptidyl to *N*-peptidyl form. These difficulties were overcome by blocking the nitrogen atom with an acetyl group which prevented the reverse transformation taking place and rendered the stage II protein susceptible to mild alkaline hydrolysis. It was necessary to find a method of acetylating stage I protein below pH 7, the pH at which the reverse transformation to *N*-peptidyl form occurred. Fortunately reaction of stage I protein with acetic anhydride in acetate buffer at pH 5 caused rapid acetylation of the amino groups; a small fraction remained unacetylated under these conditions. It is shown in Table 1 that the amount of amino N in stage II protein was almost equal to the amount present in soluble silk. It is known that the ϵ-amino group of lysine will react with nitrous acid under the conditions normally used for proteins (Chibnall, 1942) and using the value quoted by Traill (1950) for the lysine content of silk fibroin it can be calculated that about 75 % of the amino N of soluble silk is attributable to the ϵ-amino group of lysine. A possible explanation of the small but definite amino N value found in stage II protein is that the ϵ-amino groups of lysine may have failed to react with acetic anhydride at pH 5. It was found that even after several treatments with acetic anhydride the amino N of stage II protein, although diminished, did not become zero. The ease with which a base is acetylated probably depends on the amount of free amine which is present during the reaction. At a low pH a strong base would be largely in the $—NH_3^+$ form, thus making acetylation more difficult than in the case of a weak base. The ϵ-amino group of lysine would be more basic than the α-amino group of serine having its carboxyl group in peptide combination, because in the latter case the electrophilic carbonyl group is attached to the same carbon atom. This question of the reactivity of the ϵ-amino group of lysine was not considered to be very important at this stage of the investigation and has not been further studied. It seemed desirable, however, to show that a simple model substance was acetylated under the conditions used for stage I protein and it was found that *O*-benzoylserine was converted into *N*-acetyl-*O*-benzoylserine by acetic anhydride at pH 5.

Alkaline hydrolysis of the stage II protein

Titration of the aqueous solution of stage II protein with 0·01 N-potassium hydroxide revealed that a small number of acidic groups was present; some of these may have been carboxyl groups and some may have been sulphonic acid groups introduced into aromatic rings (see below). The results of this direct titration were not particularly significant.

Table 4. *Bound* —COOH *groups found by titration in three different preparations of stage II protein, compared with the bound* —COOH *groups calculated from the amino* N *in stage I protein*

(Results expressed as number of —COOH groups per 100 N atoms.)

Preparation no.	No. of —COOH groups: Calc.	No. of —COOH groups: Found by titration
1	7·2	8·1
2	7·2	8·7
3	7·2	7·4

When an excess of potassium hydroxide was added, however, a number of additional acid groups were liberated. This was demonstrated by back titration after 1·5 hr. It was concluded that saponification of the ester bonds had occurred. Two additional observations supported this conclusion: the amount of alkali consumed in the saponification process was in good agreement with the theoretical amount calculated from the amino N results on stage I protein (see Tables 1 and 4) and the alkali treatment had converted an indiffusible substance into a product of which 86 % of the nitrogen was diffusible.

There remained the possibility that acetylation of the hydroxyl group of tyrosine had occurred during the acetic anhydride treatment of stage I protein. These *O*-acetyl groups would have been readily removed on treatment with excess of alkali, and the titration results would have been invalid. Herriott (1935) has shown that when the Folin colorimetric estimation of phenolic groups is carried out at pH 8 instead of the usual pH 11, *O*-acetyltyrosine does not react. This method was used to compare the apparent tyrosine content of stages I and II proteins. The reaction was found to be dependent on concentration, and so comparison of the two proteins was made at approximately the same nitrogen concentration. It was not possible to interpret these results on a quantitative basis, because it has been shown by Reitz *et al.* (1946) that sulphuric acid causes sulphonation of tyrosine residues in proteins and that tyrosinesulphonic acid has a lower chromogenic power than tyrosine itself in the Folin estimation. It was not known how many of the tyrosine residues in stage I protein had been sulphonated. The results given in Table 5 do show, however, that very few, if any, of the tyrosine hydroxyl groups had been acetylated by the acetic anhydride treatment.

Table 5. *Estimation of tyrosine* N *in stage I and stage II proteins by the Folin colorimetric method at* pH 8

(For conditions see Experimental section.)

Substance	Total protein N taken for analysis (mg.)	Tyrosine N (% of total protein N)
Stage I protein	0·445	2·72
	0·99	2·26
	1·335	2·05
Stage II protein	0·42	2·55
	0·84	2·24
	1·26	1·97

Serine and threonine analyses carried out on stage III acetyl peptide mixture showed that almost all of the serine and threonine were in the diffusible portion (see Table 6).

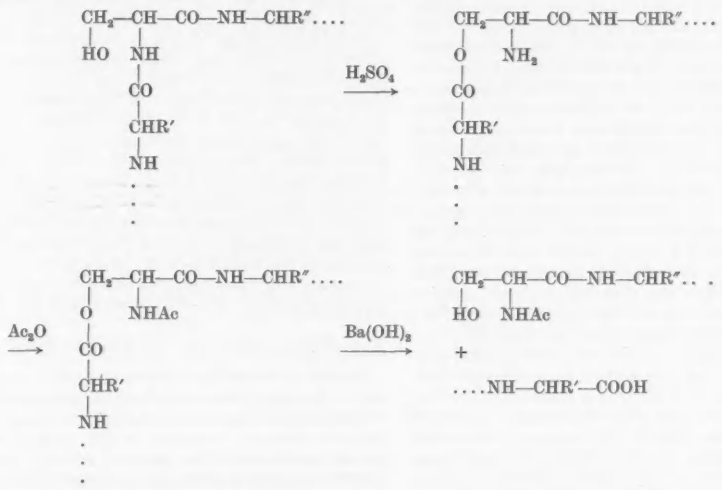
Table 6. *Distribution of serine* N *and threonine* N *in the diffusible and indiffusible fractions of stage III acetylpeptide mixture*

(Results expressed as percentages of the total N in the mixture taken for dialysis.)

	Serine N	Threonine N
Acetylpeptide mixture before dialysis	12·3	0·68
Indiffusible fraction	0·62	0·098
Diffusible fraction	11·7*	0·58*

* Value calculated by difference.

It is concluded that the degradation steps described in this paper can be formulated as shown below:



EXPERIMENTAL

Methods of analysis

Total nitrogen. This was determined by the Kjeldahl method.

Amino nitrogen. The Van Slyke method (reaction time 15 min.) was used for the manometric measurement of the nitrogen evolved on reacting the substance with HNO_2 in the Van Slyke-Neill apparatus. Several determinations were carried out on each sample. No increase in the reproducibility of the results occurred when the modified procedure of Kendrick & Hanke (1937) was used.

Alkali titrations. These were carried out in a N_2 atmosphere, using 0·01 N-KOH in methanol and bromothymol blue as indicator. To determine bound —COOH groups in stage II protein, excess of alkali was added to the protein solution and the solution back titrated after 1·5 hr.

Colorimetric estimation of phenolic groups at pH 8. This was carried out by the method of Herriott (1935) except that the colours were allowed to develop for 1 hr. at 40° before reading in the Hilger 'Spekker' photoelectric absorptiometer, using a visible light source and Ilford filter no. 608. These conditions gave more reproducible results than the 15 min. at 37° recommended by Herriott (1935). A standard reference curve was prepared for tyrosine under the same conditions.

Estimation of serine and threonine. These amino-acids were determined by the method of Rees (1946). Hydrolyses of stage III acetylpeptide mixture were carried out for 24 hr. at 115° in a sealed tube with 20 % (w/v) HCl. The hydrolysate was evaporated to dryness under reduced pressure, the residue was dissolved in water, brought to pH 6 with NaOH and made up to a suitable volume for Kjeldahl and periodate estimations.

Degradation of silk fibroin

Reaction with H_2SO_4. Commercially purified silk fibroin was washed successively with ether, ethanol, dilute acetic acid and water. It was dried for several weeks in a vacuum desiccator over NaOH, by which time the moisture content had fallen to about 5 %. The silk (2 g.) was quickly weighed and after teasing apart the fibres with a pair of tweezers it was added to 97·5 % (w/w) H_2SO_4 (40 ml.). The flask was securely stoppered to prevent entry of moisture and gently shaken by hand at 21° until the silk had dissolved. The solution was then allowed to stand at 21° for 72 hr. The solution, which was originally pale yellow, had become somewhat darker in colour, but the appearance did not suggest that appreciable charring had occurred. The solution was then cooled to −30° and to it was added Na-dried ether (600 ml.) previously cooled to −30° to −40° and the mixture thoroughly shaken. The protein precipitate was centrifuged off and washed once by centrifugation with a second portion (600 ml.) of dry ether at room temperature. To the precipitate, which was still moist with ether, was then added about 150 g. of crushed ice and the mixture well stirred. A 10 % (w/v) solution of hydrated sodium acetate was then added very carefully, with stirring to avoid local excess, until the solution had pH 3–4; it was then warmed to room temperature. A considerable amount of the protein remained undissolved in the form of a sticky gum, which was dissolved by stirring in solid NaCl in small portions. The amount of NaCl added did not appear critical, no precipitation of protein being observed when excess was added. After a final adjustment of the pH if necessary, the solution was dialysed in cellophan against distilled water saturated with CO_2, until Cl^- and SO_4^{--} were removed. The volume of the solution in the dialysis bag was allowed to increase freely during the dialysis; this seemed to assist the separation of insoluble material. At the end of the dialysis the contents of the bag were removed and centrifuged at 2000 rev./min. for 5 min. The supernatant was a pale-yellow solution which foamed readily; the sticky brown insoluble material was rejected. The recovery of N after dialysis in this and the subsequent stages in the degradation process is given in Table 3; other analyses of this material are given in Tables 1 and 5. The soluble protein is referred to subsequently as stage I protein.

Acetylation at pH 5. An aqueous solution of stage I protein (385 ml.) prepared and purified as described above and containing 0·567 mg. N/ml. was mixed with 200 ml. 4 M-acetate buffer. The buffer was prepared by mixing 15 vol. of 4 M-acetic acid solution with 35 vol. of 4 M-sodium acetate solution; at this concentration the pH of the buffer was 5·2, but on dilution with the protein solution the pH fell to 5·0. Acetic anhydride (27 ml.) was then added and the mixture shaken for a few minutes until the solution was homogeneous; it was then allowed to stand for 1 hr. at room temperature. A further portion of acetic anhydride (27 ml.) was then added and the process repeated; the amino N had then fallen to a very low value (see Table 1). It was found that no loss of amino N occurred when the mixture of protein solution and buffer was allowed to stand without addition of acetic anhydride. The acetylated protein was then dialysed against distilled water until the acidity, as determined by titration with 0·01 N-KOH, had reached a constant value, which was generally equivalent to about 0·25 ml. of 0·01 N-KOH/mg. of protein N. This method of testing for the removal of the last traces of acetate was preferable to the lanthanum nitrate test which could only be applied to the much greater volume of liquid outside the dialysis membrane. The recovery of nitrogen in this dialysis experiment is given in Table 3. Analyses carried out on the dialysed protein solution are given in Tables 1, 4 and 5. This protein is subsequently called stage II protein.

Hydrolysis of stage II protein. A solution of the acetylated protein (935 ml.) containing 0·227 mg. N/ml. was mixed with 0·1 N-$Ba(OH)_2$ (25 ml.) in an atmosphere of N_2 and the solution allowed to stand 1·5 hr. at room temperature. An exact equivalent of 0·1 N-H_2SO_4 was then added and the $BaSO_4$ removed by centrifugation. The aqueous solution was concentrated *in vacuo* to 30 ml. A small amount of $BaSO_4$ which separated during the evaporation was removed by centrifugation. This contained only 0·1 mg. of N and was rejected. The amount of $Ba(OH)_2$ required for the hydrolysis was determined by titration with 0·01 N-KOH as described above; the amount actually used was a 50 % excess over that required for saponification of all the ester groups. Analyses given in Tables 3 and 6 were carried out on the concentrated solution of acetylpeptides. This mixture is referred to subsequently as stage III acetylpeptide mixture.

Experiments with fluoro-2:4-dinitrobenzene (FDNB)

Method of reacting stage I protein with FDNB at various pH *values.* In all experiments the stage I protein was in the form of solid material obtained by dry ether washing of the precipitate produced on addition of dry ether to the H_2SO_4 reaction mixture until the washings were free from SO_4^{--}. This material was therefore contaminated with the insoluble substance which separated during the dialysis procedure described above. Amino N determinations on protein prepared in this way gave rather erratic results. This may have been due to the presence of insoluble material. An average of many determinations gave approximately the same value as that found for the stage I protein after dialysis. About 25 mg. of the protein was mixed with 1 ml. of M-acetate buffer of the required pH. A solution of 0·2 ml. of FDNB in 1·8 ml. ethanol was then added and the mixture shaken vigorously for various times and at different temperatures. The contents of the reaction vessel were evaporated to dryness under reduced pressure at room temperature. The residue was hydrolysed for 24 hr. with 6 N-HCl and the 2:4-

dinitrophenyl (DNP)-amino-acids isolated as described by Porter (1950). The mixture of DNP-amino-acids was added to a silica-gel column and the DNP-serine band separated and estimated by the method described by Porter (1950). The remaining bands were very weak by comparison with the DNP-serine band and were ignored. The results of these experiments are given in Table 2. The amount of serine N available for reaction was calculated from the amino N results given in Table 1.

Another series of experiments were carried out in which 25 mg. of protein were shaken with 1 ml. pH 5 M-acetate buffer, 0·2 ml. of FDNB and 1·8 ml. of ethanol for a certain period of time, when a second addition of buffer and FDNB, equal to the previous amounts, was made and the shaking continued for a further period. In some experiments a third portion of the reagents was added and shaking continued. The results of these experiments need not be quoted as no improvement in the yield of serine N blocked by FDNB resulted. Variations in the amount of ethanol used also brought about no improvement.

Stage I protein (25 mg.) was reacted with FDNB according to the conditions of Exp. 2 (Table 2). The DNP-protein was hydrolysed for 4 hr. with 6 N-HCl and the resulting mixture of DNP-amino-acids separated in silica-gel columns. Apart from the band due to DNP-serine there was a weaker band which was identified as DNP-glycine by comparison with authentic material in several different solvent systems. The intensity of the DNP-glycine band was about 20% of that of the DNP-serine band; other bands were very much weaker and no attempt was made to identify them. After hydrolysis under the conditions used by Porter (1950) to identify DNP-proline, no band attributable to this substance was found.

End groups in silk fibroin. Silk fibroin (0·5 g.) was cut into small pieces and shaken with a solution of $NaHCO_3$ (1 g.) in water (20 ml.), ethanol (40 ml.) and FDNB (0·33 ml.) for 24 hr. After addition of 0·33 ml. of FDNB, shaking was continued for another 48 hr. A third portion (0·33 ml.) of FDNB was then added and the shaking continued until the total reaction time was 6 days. The DNP-protein was hydrolysed for 24 hr. with 6 N-HCl and the DNP-amino-acids were examined. The mixture was very complex and no attempt was made to identify all the bands. The presence of DNP-serine and DNP-alanine was definitely established by comparing the *R* values of the bands in several solvents with the *R* values of authentic material. In a separate experiment, in which the DNP-protein was hydrolysed 4 hr. with 6 N-HCl, DNP-glycine was identified with certainty. No attempt was made to carry out these experiments on a quantitative basis. Similar results were obtained when soluble silk (Coleman & Howitt, 1947) was used.

Attempted partial hydrolysis of DNP-stage I protein. The DNP-protein was prepared under the conditions of Exp. 2 (Table 2). It was found to be completely decomposed on standing at room temperature with 0·1 N-NaOH. No DNP-peptides were produced, neither were any DNP-amino-acids found when the reaction mixture was subjected to HCl hydrolysis. When the DNP-protein was refluxed 20 min. with N-HCl and the solution extracted exhaustively with ethyl acetate a mixture of DNP-peptides was obtained. By means of silica-gel columns with $CHCl_3$ containing various amounts of butanol as the moving phase, the mixture was separated into at least eight DNP-peptides. These were separately hydrolysed. The DNP-amino-acid forming the end group was found to be DNP-serine in every case. Glycine and alanine were also found in every peptide; no other amino-acid was found. This is in agreement with recent data obtained by Drucker & Smith (1950) on the localization of serine, glycine and alanine in silk fibroin. Some preliminary experiments on methanolysis of the *O*-peptidyl bonds in the DNP-protein by treatment with methanolic HCl were not as successful as the partial acid hydrolysis.

Experiments with compounds of known structure

Rearrangement of DL-N-*benzoylallothreonine ethyl ester with ethanolic hydrogen chloride.* 1·25 g. of the ester were refluxed 1·25 hr. with 5 ml. ethanol containing 10% (w/v) HCl. The mixture was then evaporated to dryness under reduced pressure, the residue dissolved in 10 ml. of M-acetate buffer (pH 5) and extracted with ether. The aqueous layer was made alkaline with $NaHCO_3$ and again extracted with ether. The first ether extract should have contained the weakly basic oxazoline, if present, and the second contained the *O*-benzoyl ester. Both extracts gave the same picrate on addition of ethereal picric acid; this proved to be the picrate of DL-O-*benzoylallothreonine ethyl ester*, m.p. 170–171° (Found: N (Dumas), 11·6. $C_{19}H_{20}O_{11}N_4$ requires N, 11·7%) which was not depressed on admixture with an authentic sample of the substance (m.p. 169–170°: Found: N, 11·4%). This was prepared from DL-*cis*-4-carbethoxy-2-phenyl-5-methyl-Δ^2-oxazoline (Elliott, 1949) by treatment with excess of cold dil. HCl for several hours at room temperature followed by evaporation to remove excess HCl, addition of saturated sodium picrate solution and crystallization of the precipitate from ethanol. The picrate of the *cis*-ozaxoline, already mentioned, has m.p. 148–150°, that of the corresponding *trans*-oxazoline has m.p. 128–129° and that of DL-*O*-benzoylthreonine ethyl ester has m.p. 140·5° (Elliott, 1949). The structure assigned to the picrate having m.p. 170–171° is therefore probably correct.

Acetylation of DL-O-*benzoylserine at* pH 5. The amino-acid (50 mg.) was shaken with 8 ml. of M-acetate buffer (pH 5) and acetic anhydride (1 ml.). The solution became homogeneous in 1 min. The solution was allowed to stand 1 hr. at room temperature and N-HCl (5·6 ml.) was then added. The solution was evaporated to dryness and the residue extracted with ethanol. DL-*O*-Benzoyl-*N*-acetylserine (32 mg.) separated from the extracts on cooling. It had m.p. 191°. Synge (1939) gives m.p. 192–194°. (Found: C, 57·9; H, 6·0; N, 5·8. Calc. for $C_{12}H_{13}O_5N$: C, 57·4; H, 5·2; N, 5·6%.)

SUMMARY

1. Treatment of silk fibroin with concentrated sulphuric acid at 21° brought about a transfer of approximately 60% of the peptide chains linked to the nitrogen atoms of serine residues to the hydroxyl groups of these residues.

2. The resulting material was acetylated at pH 5 and then treated with excess of dilute alkali which caused hydrolysis of the *O*-peptidyl bonds and yielded a mixture of acetylseryl peptides.

3. Non-specific hydrolysis of peptide bonds may have occurred simultaneously with the rearrangement during the sulphuric acid treatment, but only to a relatively small extent.

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Studies in Carotenogenesis

3. IDENTIFICATION OF THE MINOR POLYENE COMPONENTS OF THE FUNGUS *PHYCOMYCES BLAKESLEEANUS* AND A STUDY OF THEIR SYNTHESIS UNDER VARIOUS CULTURAL CONDITIONS

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Zechmeister & Sandoval (1945, 1946) described phytofluene, a colourless polyene* showing a bluish-green fluorescence in ultraviolet light, which accompanies carotenoids* in the parts of higher plants, such as fruit and flower petals, which contain little, if any, chlorophyll. It has also been shown to occur in three classes of fungi: in the Schizomycete *Rhodotorula rubra* (Bonner, Sandoval, Tang & Zechmeister, 1946), in the Ascomycete *Neurospora crassa* (Haxo, 1949) and in the Basidiomycete *Cantharellus cinnabarinus* (Haxo, 1951). Phytofluene is more saturated than the carotenoids, containing only seven ethylenic bonds, five of which are conjugated. Porter & Lincoln (1950) consider that it is probably dodecahydrolycopene. If, as first suggested by Bonner *et al.* (1946), phytofluene is an intermediate in the biogenesis of carotenoids, it is of interest to see if it is produced by *Phycomyces blakesleeanus*, the fungus at present in use in this laboratory to study carotenogenesis. The possibility that polyenes, such as phytoene (Porter & Lincoln, 1950), which are even more saturated than phytofluene, also occur has been investigated.

In addition to the already well established occurrence in *Phycomyces* of large amounts of β-carotene and traces of α-carotene (Schopfer, 1935; Karrer & Krause-Voith, 1948; Bernhard & Albrecht, 1948; Garton, Goodwin & Lijinsky, 1951), the present investigation has shown that very small amounts of four other carotenes also occur; Bernhard & Albrecht (1948) had previously stated that traces of lycopene were present and whilst the work now reported was in progress Schopfer & Grob (1950) stated that five carotenoids exist in their strain of *Phycomyces*, but they did not discuss identification.

As colourless polyenes were found in *Phycomyces*, it was considered important to determine whether

* In this paper the term polyene refers to all the C_{40} compounds discussed, whilst the term carotenoid is used when reference is made to the coloured members of the series.

fungi which do not produce carotenoids can synthesize these related more saturated compounds. Two such fungi were examined: *Ascobolus furfuraceous*, which produces a pure white mycelium although Zopf (1890) reported that *A. pulcherrimus* synthesized lipochromes, and *Nectria cinnabarina*, which produces carotenoids only in its fruiting bodies (Kohl, 1902), but not in its mycelium when cultured on liquid media (Goodwin, 1951*a*).

When the minor polyene components of *Phycomyces* had been identified, experiments were carried out to find out how variations in cultural conditions which alter the amount of β-carotene synthesized (Garton *et al.* 1951; Goodwin & Lijinsky, 1951) affected the synthesis of the other components. The effect of the presence in the culture medium of small amounts (1/40,000) of diphenylamine was also studied, for Kharasch, Conway & Bloom (1936) noted that it inhibited chromogenesis in a number of bacteria and fungi. Turian (1950) has recently shown that in the case of *Mycobacterium phlei* it is the synthesis of carotenoids that is inhibited by diphenylamine.

A short account of a part of this work has already appeared (Goodwin, 1951*b*).

EXPERIMENTAL

Cultural conditions. Phycomyces blakesleeanus (+ and − strains) was grown under the conditions previously described in detail (Garton *et al.* 1951). In order to obtain large growths, penicillin pots containing 250 ml. of medium were used instead of the 8 oz. medicine bottles containing 15 ml. of medium used in the previous investigation. *Ascobolus furfuraceous*, *Nectria cinnabarina* and *Neurospora crassa* were cultured under the same conditions except that the medium was fortified by the addition of 0·2% (w/v) of Marmite.

Extraction and separation of carotenoids. Throughout this investigation the ether used was always freshly redistilled and the light petroleum of b.p. 40–60°. The lipid fraction was extracted from mycelia with ether according to the method of Garton *et al.* (1951) and the ether removed *in vacuo* at room temperature. The ease with which carotenoids undergo *cis-trans* isomerization on heating makes it advisable to use as little heat as possible, especially when, as here, some of the constituents readily produce numerous isomers even on slight warming.

The lipid fraction was then dissolved in a small volume (about 10 ml.) of light petroleum and chromatographed on a column of alumina. Pilot experiments showed that the lipid fraction did not need to be saponified before chromatography, but that it was necessary to carry out the separation in four stages. In each case the adsorbent was either activated or deactivated alumina or a mixture of the two; the active alumina used was Spence Grade '0', whilst the deactivated material was obtained by stirring Grade '0' alumina with an excess of methanol, allowing it to stand for 1–2 hr., removing the solvent at the pump and drying overnight at 30–40°.

Separation 1. Approximately 1·5 mg. of polyenes were fractionated on a column (15 × 1·5 cm.) containing a 4:1 (w/w) mixture of activated and deactivated alumina. The resulting chromatogram is described in Table 1.

Table 1. *First separation of* Phycomyces *polyenes on a mixture of* 4:1 *(w/w) activated and deactivated alumina, using light petroleum containing* 20% *(v/v) of ether as developer*

(The zones are numbered in order of decreasing adsorptive power.)

Zone no.	Description	Absorption maxima (in light petroleum) (mμ.)
1	Colourless; slight blue fluorescence	288, 274, ~265
2	Orange-yellow	500, 467, 437, 426, ~410
3	Orange-red. Major zone	480, 450, 423
4	Colourless; bright bluish-green fluorescence	367, 348, 331
5	Narrow; yellow-brown	446, 475
6	Colourless; blue fluorescence	340, ~280, ~240, 221
7	Colourless; non-fluorescent	296, 284, 276, 265

In additon to the zones described in Table 1 a few cultures (1–2% of total number examined) contained an ether-soluble orange-red pigment which was very strongly adsorbed at the top of the column, and which could only be eluted with ether containing 5% (v/v) of glacial acetic acid.

Separation 2. Fraction 2 (Table 1) (10–20 μg.) was chromatographed on a column (10 × 1 cm.) containing a 1:4 (w/w) mixture of activated and deactivated alumina. Table 2 shows the development of the chromatogram.

Table 2. *Resolution of fraction* 2 *(Table* 1*) on a mixture of* 1:4 *(w/w) activated and deactivated alumina, using light petroleum containing* 3–5% *(v/v) ether as developer*

(Zones are numbered in order of decreasing adsorptive power.)

Zone no.	Description	Absorption maxima (in light petroleum) (mμ.)
2*A*	Reddish-pink	505, 470, 445
2*B*	Brownish-yellow	470, 440, 416
2*C*	Pink	490, 459, 434
2*D*	Lemon-yellow	451, 423, 400, 377, 362

Separation 3. The combined fractions 6 (Table 1) from a number of experiments (representing only a very small amount of material) were chromatographed on a column (10 × 1 cm.) of active alumina. The chromatogram which developed is described in Table 3.

Separation 4. Fraction 7 (Table 1) was rechromatographed on a column (15 × 1·5 cm.) of activated alumina using 5% (v/v) of ether as developer. Absence of any property (e.g. colour, fluorescence in ultraviolet light) which would allow the development of the chromatogram to be followed easily, made it necessary to run the chromatogram 'blind'; numerous fractions were collected in an automatic fraction collector and examined spectroscopically. The first fractions (15 × 5 ml.) contained mainly material with an absorption maximum at 255 mμ. The best of these were combined as fraction 7*A*, but even in these a small amount

Table 3. *Resolution of fraction* 6 (*Table* 1) *on activated alumina using light petroleum containing* 5% (*v/v*) *of ether as developer*

(Zones are numbered in order of decreasing adsorptive power.)

Zone no.	Description	Absorption maxima (in light petroleum) (mμ.)
6*A*	Bright-blue fluorescence	~270, ~230
6*B*	Trace of green fluorescence	
6*C*	Sky-blue fluorescence	~280, ~270, ~240, 220
6*D*	Bright-blue fluorescence (major zone)	341, 326

Table 4. *Wavelengths and* $E^{1\%}_{1\,cm.}$ *values (in light petroleum) used in the quantitative determination of* Phycomyces *polyenes*

Polyene	Wavelength (mμ.)	$E^{1\%}_{1\,cm.}$	Reference
Phytoene	285	1100*	Porter & Lincoln (1950)
Phytofluene	348	1200	Zechmeister & Sandoval (1946)
α-Carotene	443	2700†	Zechmeister (1944)
β-Carotene	450	2590†	Zechmeister (1944)
γ-Carotene	459	2760†	Zechmeister (1944)
Lycopene	469	3460†	Zechmeister (1944)
ζ-Carotene	422	2500*	Porter & Lincoln (1950)
Neurosporene	439	2990	Haxo (1949)

* Calculated from data reported by Porter & Lincoln (1950).
† Calculated from graphs.

of material absorbing light maximally at 285 mμ. was present. The slower moving fraction (7*B*) contained the 285 mμ. material which also exhibited subsidiary absorption peaks at 272 and 296 mμ.

Examination of fractions

Fraction 1 (Table 1) contained mainly ergosterol (Bernhard & Albrecht, 1948) and was discarded.

Fractions 2*A–D* (Table 2). After further chromatographic purification the adsorption and spectral properties of these pigments indicated that they were probably lycopene (2*A*), neurosporene (2*B*), γ-carotene (2*C*) and ζ-carotene (2*D*). In the case of the first three they were compared spectroscopically and in mixed chromatograms with authentic specimens of the suspected pigment. Lycopene was obtained from tomatoes (e.g. Karrer & Jucker, 1949) and neurosporene and γ-carotene from *Neurospora crassa* (Haxo, 1949). In the case of ζ-carotene, no authentic sample could be obtained. It did not occur in various samples of commercial tomatoes examined (confirming the observation of Porter & Lincoln (1950) on American commercial varieties) and none could be detected in one sample of yellow maize (kindly given by J. Bibby and Son Ltd.), although it has been reported to be present in traces in some samples (White, Brunson & Zscheile, 1942).

Fraction 3 (Table 1) was β-carotene, already identified.

Fraction 4 (Table 1) corresponded with phytofluene and was compared with an authentic specimen of this polyene obtained from tomatoes according to the original method (Zechmeister & Sandoval, 1945, 1946).

Fraction 5 (Table 1) was α-carotene, already identified.

Fractions 6*A–D* (Tables 1 and 3). Little could be done with the traces available of this series of fluorescent materials, except to note that they are unsaponifiable and to record their absorption spectra (Table 3).

Fractions 7*A*, *B* (*separation* 4). Fraction 7*A* which could not be completely separated from 7*B* was not examined further. The properties of fraction 7*B* were compared with those reported for phytoene (Porter & Zscheile, 1946). An authentic specimen of phytoene could not be obtained for direct comparison. The observations of Porter & Lincoln (1950) that phytoene does not occur in ordinary commercial tomatoes was confirmed; they obtained their specimen of phytoene from specially bred crosses.

Quantitative experiments

In the experiments carried out to determine the effect of changes in cultural conditions on the synthesis of the various polyenes produced by *Phycomyces*, the separated components were dissolved in light petroleum and the amounts present determined spectrophotometrically in a Beckman instrument using the data recorded in Table 4.

In the case of phytofluene, a single chromatographic separation did not completely remove small amounts of material which absorbed light generally in the region 320–360 mμ. Using the Morton & Stubbs (1946) correction procedure, this irrelevant absorption could be corrected for by using the following equation:

$$E_{348\,m\mu.}\ (\text{corr.}) = [2E_{348\,m\mu.}\ (\text{obs.}) - E_{338m\mu.}\ (\text{obs.}) - E_{358\,m\mu.}\ (\text{obs.})]/0{\cdot}942.$$

When the amounts of lycopene, neurosporene, γ-carotene and ζ-carotene present were not sufficient to make a quantitative separation practicable they were determined together by measuring the extinction at 450 mμ. of the mixed extract in light petroleum and using an $E^{1\%}_{1\,cm.}$ value of 2500 to calculate amounts. The least accurate determinations are those for phytoene, in cultures not containing diphenylamine accurate allowance could not be made for the presence of the 255 mμ. material; the values recorded

probably represent an overestimate of the phytoene content of these cultures.

In cultures containing diphenylamine, the phytoene fractions are contaminated with diphenylamine which absorbs in the same spectral region as phytoene and thus causes analytical difficulties.

RESULTS

Identification of polyenes

The presence of lycopene, neurosporene, γ-carotene and phytofluene (fractions 2*A*, 2*B*, 2*C* and 4, respectively) in the (+) and (−) strains of *P. blakesleeanus* has been demonstrated. In each case the spectra of the suspected and authentic pigment had the same absorption maxima and, with but very minor deviations in the case of γ-carotene and neurosporene, identical shapes (Figs. 1 and 2). Further, mixed chromatograms of the suspected and authentic pigment on two adsorbents using appropriate developing solvents (Table 5) produced single zones, with no signs of separation. The other polyenes which were obtained chromatographically pure are almost certainly ζ-carotene and phytoene (fractions 2*D*, Table 2; and fraction 7, Table 1). In neither case was final proof of this established because authentic specimens were not available for direct comparison, but all other properties pointed to the identity suggested.

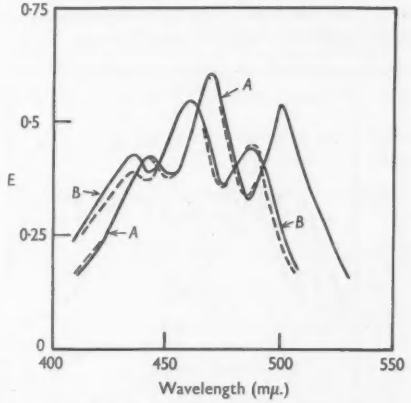

Fig. 1. The absorption spectra in light petroleum (b.p. 40–60°) of (*A*) lycopene and (*B*) γ-carotene. ——, Ex *Phycomyces*; - - - - -, an authentic specimen. In each case the *E* values of the two specimens at the wavelength of maximal absorption have been made equal.

The pigment considered to be ζ-carotene (fraction 2*D*, Table 2) had the following properties identical with those described for ζ-carotene by Nash & Zscheile (1945) and Nash, Quackenbush & Porter (1948): (*a*) it is adsorbed on an alumina column as a lemon-yellow zone just above β-carotene and just below γ-carotene; and (*b*) it has absorption maxima at 362, 377, 400 and 423 mμ. (the relative intensities at these wavelengths agreeing with those shown by ζ-carotene; see Fig. 2). The only other known carotenoid with a somewhat similar spectrum is aurochrome (Karrer & Rutschmann, 1942), but this pigment is very much more strongly adsorbed than is ζ-carotene (Nash *et al.* 1948). The one difference noted between the pigment of Nash *et al.* (1948) and the *Phycomyces* pigment is that the latter always showed a small additional absorption band at 451 mμ. This band was not recorded by Nash & Zscheile (1945), but it does not appear to be due to an impurity, for repeated chromatography on different adsorbents not only failed to eliminate it but did not alter its *E* value relative to those of the main bands.

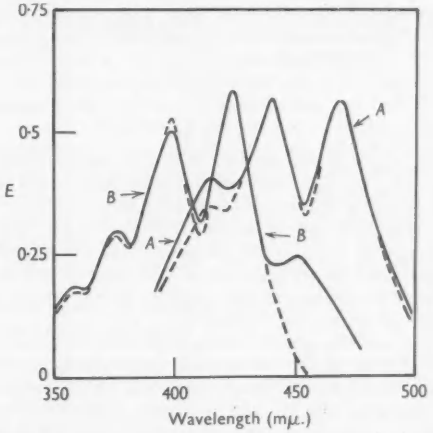

Fig. 2. The absorption spectra in light petroleum (b.p. 40–60°) of (*A*) neurosporene and (*B*) ζ-carotene. ——, Ex *Phycomyces*; - - - - -, an authentic specimen in the case of neurosporene; redrawn from the curve (in *iso*octane) of Nash & Zscheile (1945) in the case of ζ-carotene. In each case the *E* values of the two specimens at the wavelength of maximal absorption have been made equal.

Fraction 7*B* (Table 1) thought to be phytoene had the following properties, identical with those reported for phytoene (Porter & Zscheile, 1946): (*a*) it passes down an alumina column just in front of phytofluene; (*b*) it is colourless and does not fluoresce in ultraviolet light except in very concentrated solutions when it fluoresces faintly blue; and (*c*) it exhibits an absorption spectrum with its maxima in light petroleum located at 272, 285 and 296 mμ. The shape of the absorption spectrum was, however, not identical with that of phytoene, owing to the presence of small amounts of fraction

Table 5. *Adsorbents and solvent mixtures used in mixed chromatograms for the identification of the polyenes of* Phycomyces

Polyene	Adsorbent 1	Developer 1 (mixtures of light petroleum and ether*)	Adsorbent 2	Developer 2 (mixtures of light petroleum and ether)
Lycopene γ-Carotene Neurosporene Phytofluene	Al_2O_3 weakened (see p. 551)	25:1 100:3 100:3 50:1	$CaCO_3$ (analytical grade) $Ca(OH)_2$ (analytical grade)	25:1 100:3 100:3 25:1

* All concentrations are v/v.

7*A* which it was never possible to remove completely. This, combined with the fact that authentic phytoene was not available for comparison (the observations of Porter & Lincoln (1950) that no phytoene occurs in commercial tomatoes was confirmed), precluded the unequivocal identification of fraction 7*B* as phytoene. It will therefore be referred to as the 'phytoene-like fraction'.

It was thought for a long time that the presence of diphenylamine in the culture medium greatly stimulated the synthesis of phytoene by *Phycomyces* (Goodwin, 1951 *b*). Very recently, however, it has been found that the phytoene fractions from diphenylamine-treated cultures were contaminated with traces of diphenylamine itself. It happens by coincidence that the spectral and chromatographic properties of diphenylamine agree very closely with those reported for phytoene. It is, therefore, unwise to discuss the effect of diphenylamine on phytoene production by *Phycomyces*, until this unexpected analytical difficulty has been overcome. Work is in progress on this problem.

Unidentified compounds

Fractions 6*A–D* (Table 3). This group of four fluorescent compounds occurred only in very small amounts and it was not possible to examine them in any detail. They could not be identified with any known polyenes. Fractions 6*A–C* exhibited very indeterminate spectra (Table 3), whilst the major fraction 6*D* possessed a more well-defined spectrum with maxima in the near ultraviolet (Fig. 3).

Fraction 7*A* (separation 4). This material, which runs on a column slightly faster than phytoene, could not be obtained free from phytoene and has not been identified.

The strongly adsorbed orange-red pigment. This pigment, which was occasionally encountered strongly adsorbed at the top of an alumina column, could only be eluted with ether containing 5% acetic acid. It is not a carotenoid for it is extractable from an ethereal solution with aqueous sodium carbonate (5% w/v) to give a bright-green colour which rapidly changes to a deep orange; further it exhibits a spectrum quite different from that of any known carotenoid (see Fig. 3); its sporadic appearance precluded a complete investigation.

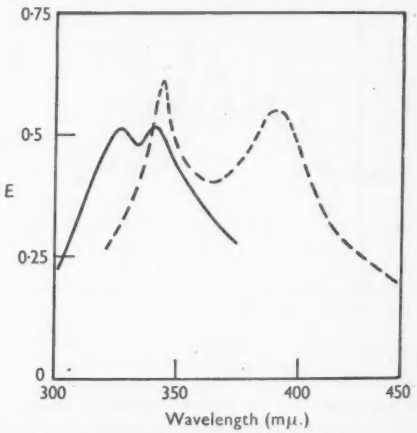

Fig. 3. The absorption spectra of (*a*) blue fluorescent material (fraction 6*D*) adsorbed on an alumina column just above phytoene, in light petroleum (b.p. 40–60°) (——); (*b*) a red pigment, very strongly adsorbed on alumina, which occurred only in 1–2% of the mycelia examined, in ether (- - - - -).

Quantitative experiments

In the experiments described in this section the (−) strain of *Phycomyces* was used unless otherwise stated. The relative amounts of the constituent polyenes (just described) were determined in a large number of mycelia cultured for 9 days on a standard medium (Garton *et al.* 1951) and the mean results obtained are collected in Table 6. Mycelia which were analysed at different stages of growth (from 3 to 22 days) showed no significant deviations from the values quoted in Table 6, and the results are therefore not recorded here. It will be seen from Table 6 that the carotenoids identified in the present work are very minor components, for, calculated on the basis of the pigments present, β-carotene represents about 95% of the total.

Table 6. *Quantitative distribution of the polyene components of the* Phycomyces *(−strain) when cultured for 9 days on a standard medium (3 % (w/v) glucose, 0·2 % (w/v) asparagine.)*

(Mean values of a large number of experiments.)

Polyene	Amount present (as % of total polyenes)
Phytoene-like fraction	13·5
Phytofluene	1·9
α-Carotene	1·9
β-Carotene	80·3
γ-Carotene	0·85
ζ-Carotene	0·35
Neurosporene	0·60
Lycopene	0·60

The production of the various polyenes was determined under the cultural conditions known to reduce (Garton *et al.* 1951) and increase (Goodwin & Lijinsky, 1951) the amount of β-carotene produced. The results, recorded in Table 7, indicate that, although the absolute amounts of the various components can vary with cultural conditions, their relative amounts show no significant variations on any of the media examined. Similarly, no changes in relative amounts are observed when *Phycomyces* was grown in the dark or when the (+) strain was used in place of the (−) strain (Table 8).

It was found that diphenylamine, when added to culture media in a concentration of 1/30,000–1/40,000, reduces the growth rate of *Phycomyces* considerably, but the final growth and lipid production are only reduced by, at most, 20 %. The surface of mature mycelia in contact with the medium is, however, colourless or slightly lemon-green with a marked green fluorescence in ultra-violet light instead of the usual bright orange-

Table 7. *Production of the polyene components by the (−) strain of* Phycomyces *cultured for 9 days on different media in the light*

(The salt and aneurin concentrations are unchanged throughout. The amounts are those produced in one penicillin pot containing 250 ml. of medium.)

	Medium used											
	3 %* glucose, 0·2 % L-asparagine		3 % xylose, 0·2 % L-asparagine		3 % fructose, 0·2 % L-asparagine		1 % glucose, 0·2 % L-asparagine		1 % glucose, 0·1 % L-leucine, 0·1 % L-asparagine		1 % glucose, 0·1 % L-valine, 0·1 % L-asparagine	
	Amounts produced											
Polyene	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total
Phytoene-like fraction	105	13·5	50	13·5	32	11·0	13	11·8	42	14·6	32	20·5
Phytofluene	15	1·9	7	1·9	6	2·1	1	0·9	6	2·1	3	1·9
α-Carotene	15	1·9	6	1·6	8	2·8	4	3·6	7	2·3	3	1·9
β-Carotene	626	80·3	300	80·7	238	82·0	89	81·0	228	79·3	113	72·5
γ- and ζ-Carotene †												
Neurosporene	19	2·4	9	2·4	6	2·1	3	2·7	5	1·7	5	3·2
Lycopene												
Total	**780**	—	**372**	—	**290**	—	**110**	—	**288**	—	**156**	—

* All concentrations are w/v. † Measured together as a single fraction.

Table 8. *Relative production of polyenes by the (−) strain of* Phycomyces *cultured for 9 days on the standard medium in the light and in the dark and by the (+) strain in the light*

(The amounts are those produced in one penicillin pot containing 250 ml. of medium.)

	Amounts produced					
	(−) strain light		(−) strain dark		(+) strain light	
Polyene	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total
Phytoene-like fraction	105	13·5	81	15·7	81	17·4
Phytofluene	15	1·9	14	2·7	15	3·2
α-Carotene	15	1·9	12	2·3	10	2·1
β-Carotene	626	80·3	388	75·2	354	76·0
γ- and ζ-Carotenes *						
Neurosporene	19	2·4	21	4·1	6	1·3
Lycopene						
Total	**780**	—	**516**	—	**466**	—

* Measured together as a single fraction.

yellow with little or no fluorescence. Quantitative analyses of the polyenes extracted from mycelia grown on media containing diphenylamine showed that, in all the cultures examined, the distribution had also undergone a profound change. Table 9 gives typical values for the polyene distribution in *Phycomyces* growing on media containing diphenylamine and shows that the synthesis of the most unsaturated polyenes (α-, β- and γ-carotenes and lycopene) has been almost completely inhibited whilst that of the more saturated compounds has been correspondingly increased. In these cultures the contribution of β-carotene to the total amount of polyenes present falls from 75 to 80 % to between 6·8 and 21 %, whilst lycopene and γ-carotene disappear completely from the mycelia. The percentage of phytofluene, on the other hand, increases from about 3 to over 50 % and ζ-carotene undergoes a similar increase (of the order of five to seven times); neurosporene is also increased but to a somewhat lesser extent. The effect on phytoene remains to be settled (see p. 554).

An examination of the two fungi *Ascobolus furfuraceous* and *Nectria cinnabarina*, which under the cultural conditions employed in this study, do not produce carotenoids, showed that they also do not produce colourless polyenes. When cultured on media containing diphenylamine, growth rate was reduced considerably, but the final amount of growth only slightly. The effect of the presence of diphenylamine in the culture media on the unsaponifiable matter produced by either fungus was, in contrast to *Phycomyces*, not appreciable. This is demonstrated in Fig. 4 which shows that the absorption spectra of the crude lipid fraction of *Ascobolus* grown on media with and without the addition of diphenylamine are indistinguishable over the range 220–310 mμ. No appreciable light absorption occurred in either fraction above 310 mμ. The absorption bands noted in Fig. 4 are probably due to ergosterol.

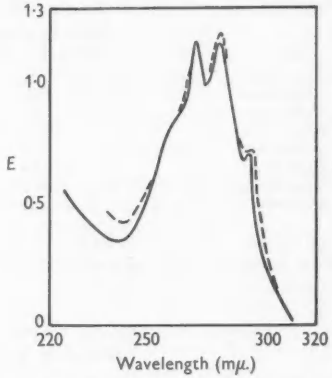

Fig. 4. The absorption spectrum of the crude lipid extract of *Ascobolus furfuraceous* cultured on a standard medium (Garton *et al.* 1951) + Marmite (0·2 % w/v), with and without the addition of diphenylamine (1/35,000). ——. Without diphenylamine; - - - - -, with diphenylamine.

Table 9. *Effect of the addition of diphenylamine* (1/40,000) *on the relative production of polyenes by* (+) *and* (−) *strains of* Phycomyces *grown for* 9 *days on various media in the light and the dark*

(The amounts are those produced in one penicillin pot containing 250 ml. The aneurin and salt concentrations of the media remained constant.)

	Medium and strain used											
	(−) strain 3 % glucose, 0·2 % L-asparagine		(−) strain 3 % glucose, 0·2 % L-asparagine		(+) strain 3 % glucose, 0·2 % L-leucine		(−) strain 1 % glucose, 0·2 % L-asparagine		(−) strain 1 % glucose, 0·1 % L-valine, 0·1 % L-asparagine		(−) strain 1 % glucose, 0·1 % L-leucine, 0·1 % L-asparagine	
	Amounts produced											
	In light		In dark		In light		In light		In light		In light	
Polyene	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total	Amount (μg.)	% of total
Phytofluene	70	58·8	109	64·9	38	87·1	24	54·6	16	55·1	20	71·4
α-Carotene	0	0	0	0	0	0	0	0	0	0	0	0
β-Carotene	25	21·0	15	8·9			3	6·8	6	20·7	3	10·7
ζ-Carotene	17	14·3	34	20·2			11	25·0	7	24·2	5	17·9
γ-Carotene	0	0	0	0	11*	12·9	0	0	0	0	0	0
Neurosporene	7	5·9	10	6·0			6	13·6	Trace	—	Trace	—
Lycopene	0	0	0	0			0	0	0	0	0	0
Total	119	—	168	—	49	—	44	—	29	—	28	—

* Measured as a single fraction.

DISCUSSION

The demonstration of the presence in *Phycomyces* of small amounts of the polyenes γ- and ζ-carotene, neurosporene, lycopene, phytofluene and phytoene is of interest from the point of view of the general distribution of carotenoids in fungi as well as from that of carotenogenesis in fungi. From the former viewpoint the present investigation extends to the Phycomycetes the presence of lycopene, neurosporene and phytofluene, which have been noted previously only in the other classes (see Goodwin, 1952, for full details). γ-Carotene, on the other hand, has already been found in the male gametangia of the sexual forms of various species of the aquatic Phycomycete, *Allomyces* (Emerson & Fox, 1940), as well as in the other classes. ζ-Carotene is not so widely distributed as are the other polyene components, for it has not been previously reported as such in any other fungus; but it now appears certain (Haxo, 1949) that the pigment occurring in one of the *Rhodotorula rubra* mutants of Bonner *et al.* (1946) is ζ-carotene. This is the first time that phytoene has been observed in a fungus, but it may be widely distributed for Goodwin (1951*a*) has recently observed it in the Basidiomycete *Dacromyces stillatus*.

The series of polyenes present in *Phycomyces* is very similar to that present in tomato fruit, the main difference being that the major pigment in *Phycomyces* is β-carotene whilst that in tomatoes is lycopene. The very great similarity between the two series of polyenes does, however, strongly suggest that the biosynthetic route may be the same in both. It is instructive, therefore, to consider the mechanism postulated by Porter & Lincoln (1950) in tomatoes as a possible route in *Phycomyces*.

ζ-Carotene is octahydrolycopene (Nash *et al.* 1948) and Porter (unpublished work quoted by Porter & Lincoln, 1950) considers that neurosporene, phytofluene and phytoene are tetra-, dodeca-, and hexadeca-hydrolycopene, respectively. Porter & Lincoln (1950) also state that tetrahydrophytoene (eicosahydrolycopene) exists in tomatoes. As little information regarding its properties is as yet available, it has not been possible to state whether or not it is present in *Phycomyces*.

From breeding studies Porter & Lincoln (1950) conclude that lycopene is the parent $C_{40}H_{50}$ carotenoid and that it is synthesized from the highly saturated compound tetrahydrophytoene, via phytoene, phytofluene, ζ-carotene and neurosporene, by the stepwise removal of four hydrogen atoms; α-, β- and γ-carotenes, etc. are then produced by the isomerization of lycopene. It is not considered likely that lycopene is the first product and that the more saturated polyenes are produced from it by reduction.

The possibility that, in bacteria, the most unsaturated polyenes are also produced from their more saturated derivatives has been envisaged by Turian (1950) who found that diphenylamine inhibited carotenogenesis in *Mycobacterium phlei*. He postulated that the inhibition occurred at the point in the chain where phytofluene or a related compound was converted into the more unsaturated polyenes, although he did not produce any experimental evidence in support of this. In fact, it has only recently been shown that phytofluene occurs in this bacterium (Goodwin, 1951*a*).

The experiments recorded here (Table 9) show conclusively that diphenylamine inhibits the production of the most unsaturated polyenes (α-, β- and γ-carotene and lycopene) and stimulates the synthesis of more saturated ones (phytofluene, ζ-carotene, neurosporene and possibly phytoene). This appears at first sight to confirm the hypotheses of Porter & Lincoln (1950) and Turian (1950). Although this interpretation of these results may be correct, it must be emphasized that another interpretation is possible. Instead of being produced stepwise one from the other, the series of polyenes could be produced in parallel syntheses from a common precursor, thus:

COMMON PRECURSOR
- →Phytoene
- →Phytofluene
- →ζ-Carotene
- →Neurosporene

} Unaffected by diphenylamine

COMMON PRECURSOR
- →α-Carotene
- →β-Carotene
- →γ-Carotene
- →Lycopene

} Blocked by diphenylamine

If the synthesis of the fully unsaturated polyenes is blocked by diphenylamine then more of the common precursor is available for the synthesis of the more saturated derivatives, which will then accumulate in the mycelium.

Tables 7 and 8 show that variations in cultural conditions which alter the β-carotene production by *Phycomyces* (Garton *et al.* 1951; Goodwin & Lijinsky, 1951) also alter to the same extent the relative amounts of the other components synthesized. The reduced synthesis of β-carotene in the dark is also reflected in the other components, and this is also the case with the (+) strain (Table 8). Thus there is no indication from these results that the decreased β-carotene production observed under certain conditions is due to the failure to convert a related

polyene into this pigment. Similarly, increased β-carotene synthesis is never at the expense of another polyene component.

The coloured components of the polyene series identified in *Phycomyces*, α-, γ- and ζ-carotenes, neurosporene and lycopene, represent only 5% of the total pigments present. As their proportion does not vary under varying cultural conditions not involving the presence of diphenylamine in the medium, no sensible errors are introduced by assuming, as Garton *et al.* (1951) and Goodwin & Lijinsky (1951) have done, that the unchromatographed pigment is β-carotene.

It is interesting to note that the hyphae of the cultures of *Phycomyces* produced in the presence of diphenylamine are still strongly positively phototropic, in spite of the fact that the β-carotene content is reduced by about thirty times. This suggests that the view of Galston (1950) that phototropic bending may be mediated through riboflavin and not β-carotene may be correct, although the small amount of β-carotene still present in 'diphenylamine cultures' may still be sufficient for this purpose. Obviously the use of these 'diphenylamine cultures' should be of great help to the plant physiologists in settling this problem.

SUMMARY

1. Phytofluene, γ-carotene, ζ-carotene, neurosporene and lycopene occur in *Phycomyces blakesleeanus*, in addition to α- and β-carotene already recorded. They represent 2·0, 0·85, 0·35, 0·6 and 0·6% respectively, of the total polyenes present. Phytoene is also probably present.

2. A red pigment, not a carotenoid, occurs in about 2% of the mycelia examined; the reason for its sporadic appearance is unknown.

3. Unidentified unsaponifiable materials include a substance with an absorption band at 255 mμ., associated with phytoene, and a small group of four fluorescent substances adsorbed on alumina between phytoene and phytofluene.

4. Variations in the cultural conditions which alter the amount of β-carotene synthesized affect the synthesis of the other component polyenes equally.

5. Addition of diphenylamine (1/40,000) to the media almost completely inhibits the production of the most unsaturated carotenoids (α-, β- and γ-carotene and lycopene), whilst stimulating the production of the more saturated components phytofluene, ζ-carotene, neurosporene and possibly phytoene.

6. *Ascobolus furfuraceous* and *Nectria cinnabarina* when cultured on liquid media do not synthesize polyenes. Diphenylamine has no effect on the unsaponifiable fraction of these fungi.

I wish to thank Prof. R. A. Morton, F.R.S., for his continued interest in this work and Dr W. Lijinsky for providing the cultures used.

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The Effect of Organic Acids, Inhibitors and Enzymes on the Respiration of *Chlorella*

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In the course of an investigation on the respiratory mechanism of the green alga *Chlorella*, experiments were conducted to study the utilization for growth in the dark of several organic acids (Eny, 1950). The effects of these organic acids on the rate of oxygen uptake and carbon dioxide evolution were observed (Eny, 1951). To elucidate further the respiratory mechanism, the action of various enzymes and enzyme inhibitors known to influence the aerobic path of carbohydrate metabolism in animal tissues was studied. The present investigation included manometric determination of the influence of cytochrome oxidase, coenzyme I (CoI), and adenosinetriphosphate (ATP) on the respiratory rate of *Chlorella*, determination of adenosinetriphosphatase (ATPase) activity, and estimation of dehydrogenase activity.

MATERIALS AND METHODS

Nutrient solutions were prepared by standard methods (Mandels, 1943), and buffering action was provided by potassium phosphate and was increased by addition of the organic acids. The pH of the system was adjusted to 5·4–5·6. The organic acids used were lactic, pyruvic, acetic, *cis*-aconitic, citric, succinic, fumaric, malic, butyric, and propionic.

Chlorella cell homogenates were prepared by a technique described by Umbreit, Burris & Stauffer (1945), utilizing a glass homogenizer, essentially a piston rotating with a vertical motion in a closely fitting glass cylinder. Examination of the homogenate showed almost entire destruction of the cells. Only 3–8% of the original count consisted of intact cells.

The strain of *Chlorella* used was originally isolated from soil by Wann (1921) and has been maintained in the laboratories of Cornell University. The strain has not been classified taxonomically (Mandels, 1943).

The cytochrome oxidase preparation was obtained from ox heart by the method of Keilin & Hartree (1938). This preparation also contains cytochromes *a*, *b* and *c*, fumarase, and several dehydrogenases. CoI was prepared from baker's yeast by the Williamson & Green (1940) method. ATP was prepared from rabbit muscle by the DuBois, Albaum & Potter (1943) method.

The manometric experiments were conducted in the dark with the Warburg apparatus according to the method described by Umbreit *et al.* (1945). The Warburg flasks were of the two side arm, centre-well and venting-plug type. They contained the cell homogenate with the necessary volume of water and nutrient solution, the organic acid intermediates, enzymes and/or inhibitors, and KOH or water in the centre well for the determination of oxygen and carbon dioxide respectively. A thermobarometer and two control flasks, one for CO_2 and one for O_2, contained all the components of the system studied. The enzymes and inhibitors were kept in the separate compartments. Results are expressed as q_{O_2} and q_{CO_2} which are μl./hr. O_2 uptake or CO_2 evolution for a volume of homogenate approximately equivalent to 10^9 cells.

Determination of ATPase activity. The test system was prepared by the method developed by DuBois & Potter (1943). The materials to be tested consisted of cell suspensions, cell homogenates or cell extracts. The cells were washed six times by repeated stirring and centrifuging with 0·02 M-KNO_3 solution (phosphate-free) before grinding or preparation of the final substrate. The ATP solution contained 565 μg. phosphorus/ml. when tested with potato apyrase (Krishnan, 1949).

The test tubes containing the system were placed in a water bath at 30° for periods from 30 min. to 3 hr. The reaction was stopped by the addition of 2 ml. of 20% (w/v) trichloroacetic acid. The tubes were centrifuged and 2 ml. samples of supernatant were taken for the determination of hydrolysed phosphorus.

The phosphorus was determined by Sumner's (1944) method. Controls were run with each series, one containing all reagents except the ATP, the other containing all reagents except the cell homogenate. The final values were corrected by subtracting the two controls.

Dehydrogenase tests. The dehydrogenase activity was estimated by measuring the degree of methylene blue (MB) reduction according to a slightly modified Thunberg method (cf. Umbreit *et al.* 1945). After a few preliminary experiments with Thunberg tubes, they were rejected because of the difficulty of preventing air leakage or of keeping the cell homogenate isolated during evacuation. Test tubes (10 ml.) (Fisher photocolorimeter) were used instead. Anaerobic conditions were maintained by placing a layer of mineral oil, 1·5 cm. thick, on top of the MB, thus isolating the system from contact with air. The cell homogenate was pipetted through the oil and the system left in the dark at 37° for varying periods of time. The degree of MB reduction was measured on the electrophotometer at 6500 A. after centrifuging the cell fragments to the bottom of the tubes.

Although readings were taken at various intervals, only those obtained at 12, 24 and 36 hr. after the experiment was set up are reported. These readings were selected on the basis of significance and convenience of comparison.

Table 1. *Changes in the oxygen uptake of* Chlorella *homogenate when treated with various substances*

(All values of q_{O_2} and q_{CO_2} were obtained by Warburg manometric experiments conducted in the dark. The figures for q_{O_2} and q_{CO_2} refer to systems to which organic acids, but no enzymes or inhibitors, were added. q_{O_2} and q_{CO_2} indicate μl./hr. of O_2 uptake and of CO_2 evolution respectively for homogenate equivalent to approximately 10^9 cells. The calculations were made on a 4 hr. basis. Each figure represents the average of three determinations with the same batch of homogenate. The percentage stimulation or inhibition varied within a range of $\pm 4\%$. Values were calculated on the O_2 uptake on a 2 hr. basis. + Indicates stimulation; − indicates inhibition; 0 indicates no effect. The ATP solution contained 565 μg. of phosphorus/ml. A 1% (w/v) Co I solution was used.)

Organic acid	Concn. (M)	q_{O_2}	q_{CO_2}	Percentage stimulation or inhibition with added substances: Cytochrome oxidase (0·4 ml.)	Co I (0·4 ml.)	ATP (0·4 ml.)	Sodium malonate (0·02 M)	KCN (0·004 M)	Sodium azide (0·001 M)
Lactate	0·05	632	987	+79	+72	0	+ 7	−73	−78
Pyruvate	0·05	676	178	−12	−18	0	+10	−59	−65
Acetate	0·05	952	611	−16	0	0	+13	−64	−68
Aconitate	0·03	810	496	0	0	0	+ 6	−50	−64
Citrate	0·02	335	121	−20	0	0	+10	−61	−64
Succinate	0·03	484	382	− 8	0	0	+ 8	−46	−57
Fumarate	0·03	700	486	−28	0	0	+ 2	−54	−70
Malate	0·05	410	344	+74	+93	0	+ 6	−68	−72

RESULTS

Manometric experiments

Table 1 gives the q_{O_2} and q_{CO_2} for *Chlorella* homogenate with addition of individual organic acids and without enzyme or inhibitor, and the percentage stimulation or inhibition produced by the addition of several substances. Cytochrome oxidase and Co I both stimulated the respiration of the *Chlorella* homogenate in the presence of sodium lactate and sodium malate. Cytochrome oxidase did not affect respiration in the presence of aconitate and slightly inhibited it with the other acids.

Potassium cyanide inhibited the oxygen uptake in varying degrees according to the organic acid present in the system. The largest inhibitions were produced in the presence of lactate and malate. Respiration was 70% inhibited by 0·001 M-azide and completely stopped by higher concentrations.

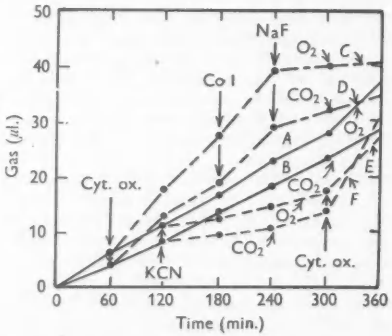

Fig. 1. Cumulative effects as studied in a Warburg manometric apparatus, of cytochrome oxidase, Co I, and 0·02 M-NaF (curves *C* and *D*) and of 0·004 M-KCN and cytochrome oxidase (curves *E* and *F*) on the respiration rate (O_2 uptake and CO_2 evolution) of *Chlorella* homogenate on malate. Curves *A* and *B* represent the controls with 0·05 M-malate. Each flask contained a total liquid volume of 5 ml. and the equivalent of $1{\cdot}4 \times 10^7$ cells. The concentration of Co I was 0·04% (w/v). 0·2 ml. of cytochrome oxidase preparation was used.

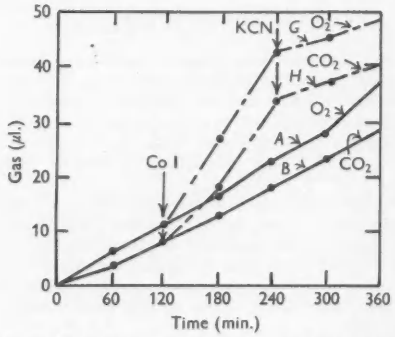

Fig. 2. Co I stimulation (0·04% w/v) and KCN inhibition (0·004 M) as studied in a Warburg apparatus, of the respiration of *Chlorella* homogenate on malate. Curves *A* and *B* represent the controls with 0·05 M-malate. Each flask contained a total liquid volume of 5 ml. and the equivalent of $1{\cdot}4 \times 10^7$ cells.

Since malonate specifically inhibits succinic dehydrogenase, the use of malonate with succinate should stop the formation of fumarate and inhibit the respiration of *Chlorella* if the oxidation requires succinic dehydrogenase as the flavoprotein. Actually malonate produced some increase of oxygen uptake, not only with succinate but with all the acids used.

A fairly large concentration of fluoride (0·02 M), was needed to produce inhibition but when reached, the inhibition was complete. Pyrophosphate inhibition was effective in the same general manner at 0·01 M concn., whilst even at the very low concentration of 0·0001 M, iodoacetate was completely toxic to the respiratory system.

No change in the rate of respiration was observed when quantities varying from 0·1 to 0·5 ml. of an ATP solution containing 565 μg. of phosphorus/ml. were used.

Since the effects of the enzymes and inhibitors tested showed with malate as well as or better than with the other organic acids, they have been charted graphically to typify the results. Figs. 1 and 2 show the changes in respiratory rate produced by the addition of Co I, cytochrome oxidase and several inhibitors to *Chlorella* homogenate in dilute nutrient solution containing 0·05 M-malate. In both figures, curves *A* and *B* represent respectively the oxygen uptake and carbon dioxide evolution without tipping enzymes or inhibitors from the side arms of the flask.

Determination of ATPase activity

To determine whether the results obtained with ATP were due to the absence of a hydrolysing enzyme or to the ineffectiveness of ATP in the metabolism of *Chlorella*, estimations of the hydrolytic enzyme of ATP were made using cell suspensions, cell extracts, or cell homogenates. The results obtained with the suspensions and extracts were not significant. Those obtained with the homogenates are shown in Table 2.

The recovery of phosphorus generally increased with the amount of homogenate in the test system and calcium chloride gave little noticeable activating effect. The time required by the *Chlorella* homogenate to produce detectable amounts of phosphorus through ATP hydrolysis was much longer than that generally necessary for animal tissues. However erratic the phosphorus recovery, the results are positive and indicate the presence of an enzyme producing some hydrolysis of ATP. Several enzymes of this type have been isolated from plant material, one of the best sources being potato (Krishnan, 1949).

Dehydrogenase tests

In animal tissue, the presence of cyanide is necessary to obtain the test for lactic acid dehydrogenase (Straub, 1940), as cyanide binds the pyruvate which is formed. Otherwise the accumulation of pyruvate would reverse the direction of the reaction and prevent the reduction of MB. If pyruvate is added to the system after the MB is reduced, the blue colour returns, showing that the MB is re-oxidized and that the reaction goes toward lactate.

In one of the tests conducted with *Chlorella* homogenate, the cyanide was omitted. The reaction continued, however, and reduction was nevertheless obtained. Pyruvate was then added to the system, but the blue colour did not reappear. Apparently the system was different from that of animal tissues and it was decided to further utilize the MB technique to study the possible dehydrogenases involved. The method gives an approximate relative rate of oxidation of the acids by the dehydrogenase system.

The colour readings of the test system are given in Table 3. The times appear unduly long, but since duplicates gave closely similar results it would seem that the results are due to the cell homogenate processes rather than to bacterial contamination. All the acids decreased the reduction time of MB, which indicates that they produce a rise in dehydrogenase activity by stimulating the respiration rate.

Pyruvic acid reduced MB more quickly than any of the other acids, and addition of Co I and cyto-

Table 2. *Estimation of ATPase in* Chlorella *homogenate*

(The complete test system (DuBois & Potter, 1943) contained cell homogenate, 0·1 M-acetate buffer pH 6·5, 0·04 M-$CaCl_2$ as activator and ATP. 0·4 ml. of the ATP solution represents 226 μg. of phosphorus. The phosphorus was analysed by the technique of Sumner (1944).)

Sample no.	Buffer (ml.)	Cell homogenate (ml.)	$CaCl_2$ (ml.)	ATP (ml.)	Time (hr.)	Phosphorus recovery (μg.)
1	0·05	2·0	0·25	0·4	1	40
2	0·05	2·0	0·25	0·4	1	24
3	0·05	2·0	0·00	0·4	1	28
4	1·00	1·0	0·25	0·4	1	19
5	1·00	1·0	0·25	0·4	1	21
6	1·00	1·0	0·00	0·4	1	15
7	0·20	3·0	0·25	0·4	3	58
8	0·20	3·0	0·25	0·4	3	43
9	0·20	3·0	0·00	0·4	3	32
10	0·50	2·0	0·25	0·4	3	57
11	0·50	2·0	0·25	0·4	3	17
12	0·50	2·0	0·00	0·4	3	36

chrome oxidase to the pyruvate system did not activate the MB reduction.

Table 3. *Dehydrogenase tests*

(The tests were conducted by a slightly modified Thunberg technique. Each tube contained 1 ml. of 0·02% MB, 1 ml. of 0·02 M-phosphate buffer pH 6·5, organic acids to 0·05 M final concentration, inhibitors to concentrations listed, or 0·5 ml. of 1·0% (w/v) Co I solution, or 0·5 ml. of cytochrome oxidase preparation, water to adjust to 6 ml., 1·5 cm. layer of mineral oil, 1 ml. of *Chlorella* homogenate pipetted through the oil.)

Substrate	Colorimeter readings at 0 hr.	12 hr.	24 hr.	36 hr.
Lactate	76	29	8	1·0
Pyruvate	76	10	4	1·2
Acetate	76	43	18	1·8
Aconitate	76	47	18	1·2
Citrate	76	55	28	1·6
Succinate	76	59	30	2·5
Fumarate	76	34	12	1·5
Malate	76	38	16	2·0
No acid	76	70	54	37·0
Lactate + cytochrome	76	18	8	2·0
Pyruvate + cytochrome	76	27	13	1·0
Acetate + cytochrome	76	70	70	3·6
Aconitate + cytochrome	76	50	18	2·4
Citrate + cytochrome	76	72	60	2·8
Succinate + cytochrome	76	61	29	3·8
Fumarate + cytochrome	76	75	73	1·8
Malate + cytochrome	76	28	9	1·5
No acid + cytochrome	76	55	38	12·3
Lactate + Co I	76	15	6	1·0
Pyruvate + Co I	76	36	9	1·0
Acetate + Co I	76	45	20	1·6
Aconitate + Co I	76	48	19	1·8
Citrate + Co I	76	54	23	1·7
Succinate + Co I	76	59	29	2·0
Fumarate + Co I	76	38	10	1·3
Malate + Co I	76	35	7	1·8
No acid + Co I	76	56	40	38·0
KCN (0·004 M)	76	64	52	13·4
Azide (0·001 M)	76	70	70	70·0
Fluoride (0·02 M)	76	70	70	70·0
Malonate (0·02 M)	76	52	43	11·0
Pyrophosphate (0·01 M)	76	67	57	55·0
Iodoacetate* (0·0001 M)	76	1	1	1·0
No substrate	76	70	54	27·3

* Discoloration was caused by the reaction of MB with iodoacetate, forming a brown precipitate.

Table 3 also shows the effect of cytochrome oxidase when added to the various acids in the dehydrogenase test systems. The reduction of MB with lactate and malate occurred faster when cytochrome oxidase was present in the system than when absent. The reason for the large inhibiting action of cytochrome oxidase in the presence of fumarate, acetate or citrate is not clear. It is possible that these acids formed some inhibitory complex with the cytochrome. (The preparation of cytochrome is not a pure system and may contain some substances reacting with the acids.) Co I did not produce this inhibition.

Enzyme inhibitors were used in the dehydrogenase tests without acid substrates. The results are shown in Table 3. The amount of inhibition was highest with iodoacetate. With 0·0001 M-iodoacetate, whether or not homogenate was added to the system, the MB was irreversibly discoloured within 5 min. and formed a heavy precipitate. Azide, fluoride and pyrophosphate were very efficient inhibitors. The inhibition produced by potassium cyanide started to decrease after about 6 hr. Complete MB reduction occurred almost simultaneously with that of the control (after about 36 hr.). Malonate failed to produce inhibition.

Inhibitors were also used in the presence of acids in the dehydrogenase tests. Their effects were similar to those reported in the manometric experiments. For instance, the use of malonate together with succinate did not decrease the respiration rate. The reduction of MB occurred in about the same time as when no malonate was added.

DISCUSSION

The presence of Co I and cytochrome has been demonstrated in various plant materials. Euler & Nilsson (1931) showed that Co I was present in boiled extracts from jute seeds. Holmberg (1934) prepared a coenzyme from peas that had the same action as Co I in reducing MB in the presence of malate or lactate. Meeuse (1948) extracted a mixture from pea seedlings showing a high cytochrome oxidase activity. In the present investigation it was shown that Co I and cytochrome oxidase increased the rate of lactate and malate oxidation in *Chlorella*. This suggests that these enzymes may be involved in the oxidation of lactate and malate or they may participate in the enzyme system naturally occurring in the cells.

Co I and cytochrome did not affect the oxidation of pyruvate, and since the dehydrogenase tests showed that this acid was oxidized faster than any other, it would appear that the oxidation is more direct than that described in the tricarboxylic acid cycle. This points toward a mechanism of oxidation involving a pyruvic dehydrogenase and flavin-adenine dinucleotide instead of the Co I-cytochrome-lactic dehydrogenase system. Lipman (1940) found that in *Lactobacillus delbrückii* oxidation of pyruvate by pyruvic dehydrogenase could take place under both anaerobic and aerobic conditions.

Emerson (1927) found that 0·0001 M-hydrogen cyanide inhibited about 60% of the respiration of a strain of healthy *Chlorella pyrenoidosa* cells which had been grown with glucose, but that the inhibition was small and temporary when lactic acid was used in the respiratory flasks with starved cells.

Warburg (1919) found a stimulation of respiration by cyanide in a strain of *C. pyrenoidosa*. If the

experiments of Emerson and Warburg were conducted with the same strain, it would appear that an alternative respiratory mechanism is available in that particular *Chlorella* strain. In the present experiments, 0·004 M-potassium cyanide produced a decrease in the rate of oxygen uptake in all cases. This inhibition reached about 70 % with lactate and malate. It was possible to reverse the inhibition by adding cytochrome oxidase (Fig. 2).

It has been shown that potassium cyanide acts not only on cytochrome but also on several other oxidases (cf. Porter, 1946). Because of these other inhibitions, James (1946) stated, 'It cannot be assumed...that any respiration of a plant tissue inhibited by cyanide is necessarily due to the cytochrome system.' But since cytochrome oxidase produced an activation of the lactate oxidation, it is at least likely that this system exists in *Chlorella* cells.

Kohn (1935) noted that both respiration and photosynthesis of *C. pyrenoidosa* were inhibited by 0·001 M-iodoacetate. The same concentration of iodoacetate produced a permanent inhibition with the *Chlorella* strain used in the present investigation. No specific effect could be studied with this inhibitor. Similarly other inhibitors, such as fluoride and pyrophosphate, produce a general toxicity and often give insignificant results (Bartlett & Guzman-Barron, 1947).

Though malonate characteristically inhibits succinic dehydrogenase it did not inhibit the oxygen uptake or the rate of MB reduction. This result does not fit in the scheme of the tricarboxylic acid cycle. Henderson & Stauffer (1944) reported that tomato roots also resist malonate poisoning. If succinic dehydrogenase is present, it is probably not the only path of succinate oxidation, as some alternate mechanism remains active in spite of malonate. This might fit with the theory advanced by Kalnitsky, Wood & Werkman (1943) on a succinate formation through the oxidative coupling of two acetates and the presence of a separate mechanism for the formation of C_3 and C_4 acids in *Chlorella*.

The utilization of high-energy phosphate bonds by cells has given an explanation for the reversal of the carbohydrate breakdown cycle in animal tissues (Kalckar, 1942). It was shown by Lepage & Umbreit (1943) that the energy release of sulphur bacteria is coupled with the formation of ATP from inorganic phosphorus and that the high-energy phosphate bonds can be utilized to fix carbon dioxide in the dark for chemosynthesis.

The present experiments with ATP gave no evidence that phosphorylated compounds were utilized by *Chlorella* in the dark. Some indication was obtained that an enzyme capable of hydrolysing ATP is present.

The path of phosphorus in the strain of *Chlorella* used in the present work may be different from that in most animals and bacteria. Emerson, Stauffer & Umbreit (1944) were unable to identify the phosphorylated compounds present in other *Chlorella* cells with those normally present in most animal tissues and bacteria.

SUMMARY

1. The effect of several enzymes, inhibitors, and adenosinetriphosphate, together with organic acids, on the rate of oxygen uptake of *Chlorella* homogenate was studied by manometric experiments conducted in the dark. An investigation of the dehydrogenase system was also conducted by a slightly modified Thunberg technique.

2. Cytochrome oxidase and coenzyme I, in the presence of malate and lactate, produced an increase in the rate of oxygen uptake and also shortened the time of methylene blue reduction. Potassium cyanide inhibited this stimulation. These effects suggest that malate and lactate are oxidized in *Chlorella* in the same manner as in animal tissues.

3. Malonate did not produce an inhibition of respiration with or without succinate. This might indicate that succinic dehydrogenase is not the only possible path to fumarate formation and points toward a separate mechanism for the formation of C_3 and C_4 acids.

4. The comparatively rapid oxidation of pyruvate by *Chlorella* in the presence of methylene blue strongly suggests that pyruvate oxidation takes place through a pyruvic-dehydrogenase system.

5. The respiration rate of *Chlorella* was not affected by the addition of various amounts of adenosinetriphosphate to systems with or without organic acids. Some hydrolysis of adenosinetriphosphate was detected.

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The Reversible Dissociation of Insulin and its Minimum Molecular Weight

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In two previous papers (Gutfreund, 1948*a*, *b*) it has been shown that the molecular weight of insulin is dependent upon pH, temperature and protein concentration of the solution. In neutral solutions of moderate protein concentration insulin appeared to be homogeneous with respect to molecular weight, M = 48,000 (Gutfreund & Ogston, 1946; Gutfreund, 1948*a*). Under optimum conditions for dissociation (pH 2·6) a steep extrapolation to zero concentration of osmotic pressure against insulin concentration gave a value of 12,000 for the molecular weight. From this evidence, as well as from crystallographic and chemical information, it was concluded that insulin in solution consists of aggregates of loosely bound sub-units of molecular weight 12,000. It is not claimed that aggregation into molecules containing four sub-units is necessarily an upper limit.

More recently two papers gave an account of ultracentrifugal and diffusion studies on insulin solutions, and the authors drew different conclusions from their results. Pedersen (1950) believes that the molecular weight of insulin is 42,000 and that molecules of that size dissociate into two sub-units of molecular weight 21,000. His data would, however, be quite consistent with the hypothesis that under varying conditions of solution the mean molecular weight can have any value from 12,000 to 48,000 or more. Fredericq & Neurath (1950) found that in dilute phosphate buffer of pH 2·65, insulin dissociates into sub-units of molecular weight 6000, this value being calculated from diffusion and sedimentation constants extrapolated to zero concentration. This extrapolation is, however, very steep, and since measurements of the diffusion constant in dilute solutions are likely to give values which are too high, the extrapolated value of the molecular weight is likely to be too low.

The present paper is confined to the determination of the molecular weight of the sub-unit (minimum molecular weight) into which insulin can be dissociated reversibly. It has already been emphasized by Gutfreund (1948*b*) that the study of the effect of pH, temperature and protein concentration upon the aggregation of the sub-unit of insulin should be complemented with an investigation of the influence of salt concentration on this phenomenon. The present paper describes this further investigation, and shows how it confirms and clarifies the author's previous findings.

Table 1. *Composition of buffers*

Series	Molar composition of buffer	μ	pH
A	H_3PO_4:0·0033, NaH_2PO_4:0·01	0·013	2·69
B	H_3PO_4:0·017, NaH_2PO_4:0·05	0·067	2·65
C	H_3PO_4:0·033, NaH_2PO_4:0·10	0·133	2·60
D	H_3PO_4:0·110, NaH_2PO_4:0·33	0·44	2·56
E	H_3PO_4:0·017, NaH_2PO_4:0·05, NaCl:0·20	0·167	2·56
F	H_3PO_4:0·033, NaH_2PO_4:0·10, NaCl:0·40	0·533	2·55
G	Na_2HPO_4:0·0218, citric acid:0·089	0·213	2·60
H	Na_2HPO_4:0·0218, citric acid:0·089, NaCl:0·20	0·413	2·60

EXPERIMENTAL

The osmotic pressure of insulin solutions was measured by the simple method of Adair (1925). The osmometers were set up in boiling tubes holding 150 ml. of diffusate and left to equilibrate in a cold room at 3°. Collodion membranes holding about 4 ml. of protein solution were used, and the pressure was recorded directly from the height of the solution in the capillary tube mounted in the membrane above the level of the diffusate. Corrections for capillarity and density of the solution must be applied to these measurements. Since there is some danger that material of low molecular weight, such as the sub-unit of insulin, may diffuse through the membrane, only results from experiments which gave constant pressure readings over a period of 24 hr. were used. It was found that membranes prepared by the method of Adair (1925) are impermeable to material of molecular weight 12,000 if five coats of collodion solution (10 g. of Imperial Chemical Industries Ltd. Nitrocellulose HL 30/40 into a mixture of 100 ml. dried ether, 100 ml. dried ethanol and 4 ml. of ethylene glycol) are applied to the glass mould.

The insulin concentration in the solution, after osmotic equilibration, was determined by the micro-Kjeldahl procedure with all the precautions recommended by Chibnall, Rees & Williams (1943). A value of 15·6 % was taken as the N content of dry ash-free insulin.

Measurements of the sedimentation velocity were carried out in the Svedberg oil-turbine ultracentrifuge at Oxford. The diagonal schlieren optical system of Philpot (1938) was used for the observation of sedimenting boundaries. The procedures recommended by Cecil & Ogston (1948) were followed for the observations, measurements and computations of sedimentation constants. The solvent buffers were made up to the composition given in Table 1 and the pH values were checked with a glass electrode. For all but a few experiments specially marked, five times recrystallized Zn insulin from Boots Pure Drug Co. Ltd. was used. Dr H. Neurath pointed out to the present author that some of the differences in results obtained may be due to variation in the different samples used. Unfortunately, samples of the particular Eli Lilly insulin used by Dr Neurath were not available. Some experiments were therefore carried out with a small portion of a sample of Eli Lilly T2536 Zn insulin, which was made available by Mrs D. Hodgkin.

RESULTS AND ANALYSIS OF OSMOTIC PRESSURE MEASUREMENTS

The results of measurements of the osmotic pressure of ideal or very dilute solutions can be used directly for the calculation of the mean molecular weight M from the equation

$$M = \frac{RT}{P/C}, \qquad \text{(i)}$$

where R is the gas constant, T the absolute temperature, P the osmotic pressure in cm. of water and C the protein concentration in g./l. Deviations from the ideal laws at finite protein concentrations have been discussed in a variety of reviews (e.g. Gutfreund, 1950). The procedure of measuring the osmotic pressure of a series of solutions over a range of protein concentration and of calculating the molecular weight from P/C extrapolated to $C=0$ gives reliable results if (1) the mean molecular weight is independent of protein concentration, and (2) the partial pressure due to Donnan effects is not very great.

In the present study the maximum protein concentration of solutions used for osmotic pressure measurements was about 10 g./l. and most of the deviation normally encountered in thermodynamic studies of solutions of large molecules are negligible under such conditions. The anomalies which have to be dealt with in the study of insulin in acid solutions are due to the dependence of the molecular weight of insulin on protein and salt concentration. If the latter effect is studied in buffer solutions of low ionic strength, the Donnan effect becomes very marked. The buffer solutions used ranged in pH from 2·55 to 2·69. Assuming that all the basic groups and none of the acidic groups are ionized in this range of pH, insulin would have one positive charge per molecular weight of 1000 (see review of chemical analysis, Sanger, 1949). The partial osmotic pressure P_i due to the unequal distribution of the $^-H_2PO_4$, H^+ and Na^+ ions of the principal buffer system used, can be calculated from the Gibbs-Donnan membrane equilibrium equation

$$[H^+ + Na^+]_S \times [^-H_2PO_4]_S = [H^+ + Na^+]_D \times [^-H_2PO_4]_D, \qquad \text{(ii)}$$

where the subscripts S and D stand for solution and diffusate respectively. Electric neutrality in the protein solution requires that

$$mZ^+ + [H^+ + Na^+]_S = [^-H_2PO_4]_S, \qquad \text{(iii)}$$

where mZ^+ is the product of the molarity of the protein and the charge valence. The volume of the diffusate is taken as sufficiently large for the equality $[H^+]_D + [Na^+]_D = [^-H_2PO_4]_D$ to hold. From the above relations it is clearly possible to derive an expression for P_i as a function of mZ^+, $[H^+ + Na^+]_D$ and $[^-H_2PO_4]_D$. Details of such derivations are given in two recent reviews, Adair (1947) and Gutfreund (1950). In Fig. 1 a plot of P_i against

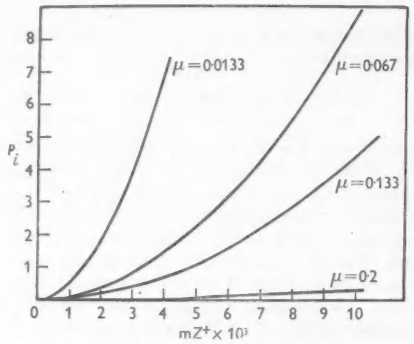

Fig. 1. Plot of P_i in cm. of water against mZ^+ for different ionic strength of 1:1 valence type buffer mixtures.

mZ^+ is shown for three different buffer compositions. The graphs were computed from the equation

$$P_i = RT\{\sqrt{((mZ^+)^2 + 4[^-H_2PO_4]^2\} - 2[^-H_2PO_4]\}. \quad \text{(iv)}$$

Strictly speaking, the osmotic activity of the ions and not their concentrations should be taken for the above calculations. Corrections by means of osmotic coefficients cannot easily be applied to mixtures of low salt concentration, since insufficient data are available. No osmotic activity correction of P_i has been attempted in the present case, since it would not be likely to reduce the value of P_i by more than 10%.

Table 1 shows the composition of the various buffer solutions used for the osmotic pressure measurements, together with their pH and ionic strength μ calculated from the equation $\mu = \frac{1}{2}\Sigma mv^2$, where m is the molarity and v the valence of each kind of ion in the solution. The letters A to H will be used throughout to indicate the type of buffer used for the experiment.

The first two series of osmotic pressure measurements were carried out on insulin solutions in buffers B and C. The results of these experiments are recorded in Figs. 2–4. It can be seen that the difference in the results obtained from these two series is only due to the greater values for P_i in the more dilute phosphate solutions. The values for P_i were estimated from the graphs in Fig. 1 on the basis of insulin having one positive charge per unit of molecular weight 1000 at pH 2·6. The slopes of the lines representing the corrected plots of $(P - P_i)/C$ against C give a measure of the increase of the mean molecular weight with insulin concentration. The calculation of the molecular weight extrapolated to zero concentration is done by numerical substitution in Eqn. (i), which gives at 3°: $M = \dfrac{23400}{P/C}$. (P in cm. of water and C in g./l.) The four graphs shown in Figs. 2–4 extrapolate to $P/C = 2$ and in spite of the

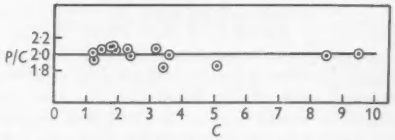

Fig. 2. Plot of P/C against C, where P is the total osmotic pressure in cm. of water and C insulin concentration in g./l. of buffer B.

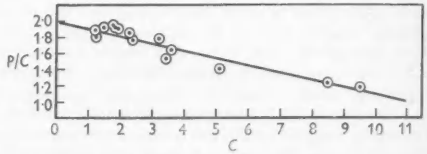

Fig. 3. Plot of $(P - P_i)/C$ against C, where $(P - P_i)$ is in cm. of water and C the insulin concentration in g./l. of buffer B.

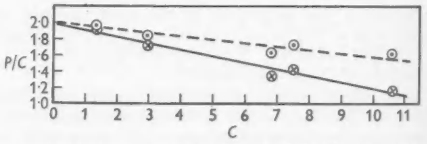

Fig. 4. Plot of $(P - P_i)/C$ (⊗) and P/C (⊙) against C, where P and P_i are in cm. of water and C the insulin concentration in g./l. of buffer C.

uncertainties of the extrapolation this value is accurate to ± 0·1. Comparison of these experiments with those previously reported (Gutfreund, 1948*b*) showed that a decrease in salt concentration affects the aggregation of insulin at finite protein concentrations. As can be seen from Fig. 1, when $\mu = 0{\cdot}0133$ the corrections for P_i become very large and the results become less accurate. Values obtained for $(P - P_i)/C$ from measurements on insulin solutions in buffer A fell within the same limits as those recorded in Fig. 3 for the measurements in buffer B. Osmotic pressure measurements in buffer H were carried out for comparison with a previous study (Gutfreund, 1948*b*) and the results (see Fig. 5) are in very good agreement with those previously reported. Experiments carried out in buffer G are also plotted

in Fig. 5. Both these graphs again extrapolate to $P/C = 2$ at $C = 0$.

In Fig. 6 isolated experiments are recorded to give further information about the effect of an increase in salt concentration upon the mean molecular weight of insulin. The peculiar solubility properties of insulin limit the range of protein and salt concentration. These experiments were designed primarily to find the optimum conditions for reversible dissociation into sub-units and only to a lesser extent to obtain any information about the mechanism of aggregation.

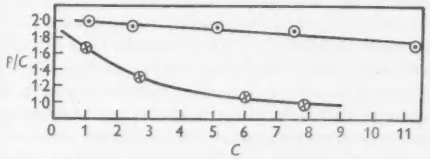

Fig. 5. Plot of P/C against C, where P is the osmotic pressure in cm. of water and C the insulin concentration in g./l. of buffer G (⊙) and H (⊗) respectively.

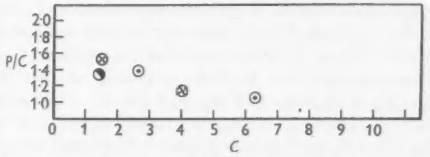

Fig. 6. Plot of P/C against C, where P is the osmotic pressure in cm. of water and C the insulin concentration in g./l. of buffer D (⊗), E (⊙) and F (◑) respectively.

The osmotic pressure of 0·23 and 0·125 % solutions of Eli Lilly insulin in buffer B was measured and the values for $(P-P_i)/C$ were 1·88 and 1·85 respectively. This is in fair agreement with the results of measurements on Boots insulin used for the rest of the experiments.

RESULTS OF ULTRACENTRIFUGE MEASUREMENTS

Three solutions of insulin in buffer B were examined in the ultracentrifuge. They contained (1) 0·5 % Boots insulin, (2) 0·25 % Boots insulin, and (3) 0·22 % Eli Lilly insulin, respectively. Cecil & Ogston (1948) suggested the graphical computation of S_{20} from a plot of $\Sigma(\Delta x)$ against $\Sigma(\Delta t')$, where Δx is the movement of the centre of the boundary during time Δt and $\Delta t' = \Delta t(\eta_{20}/\eta_\theta)\, X\omega^2$; η_{20} and η_θ are the viscosities of water at 20° and at the rotor temperature respectively; X is the mean distance of the boundary from the centre of rotation and ω the mean angular velocity. S_{20} the sedimentation constant in cm./sec./dyne at 20° can then be calculated from

$$S_{20} = \Sigma(\Delta x)/\Sigma(\Delta t'). \qquad (v)$$

For the best use of all the information the sedimentation constant should be computed from the regression of x on t' by the following equation

$$S_{20} = \frac{\Sigma\{x(t'-\bar{t}')\}}{\Sigma(t'-\bar{t}')}.$$

Fig. 7 shows the plot of $\Sigma(\Delta x)$ against $\Sigma(\Delta t')$ for the second experiment, a 0·25 % solution of Boots insulin. The sedimentation constant calculated from this run $S_{20} \times 10^{13} = 1{\cdot}65 \pm 0{\cdot}07$ (S.D. from the regression coefficient). The other results were for the first run $S_{20} = 1{\cdot}62 \times 10^{-13}$ and for the third run $S_{20} = 1{\cdot}67 \times 10^{-13}$. All these values are corrected to sedimentation in water at 20°, and Cecil & Ogston's (1948) correction factor for the thermocouple has also been introduced.

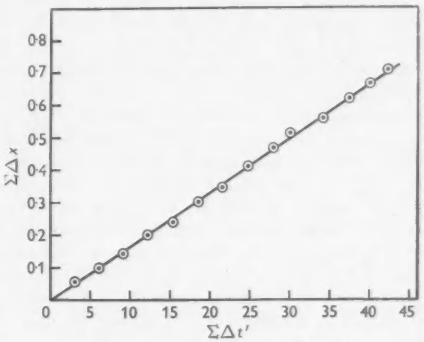

Fig. 7. Plot of $\Sigma(\Delta x)$ in cm. measured on the photographic record against $\Sigma(\Delta t')$ in cm. measured on the photographic record $\times 10^{-11}$/sec. for sedimentation velocity run on 0·25 % insulin in buffer B. (See Eqn. v for explanation of symbols.)

The sedimentation diagrams obtained showed traces of faster sedimenting material, but there was no indication of any more slowly sedimenting material on the buffer side of the boundary. The latter effect would be observed if the main boundary peak represents material which undergoes further dissociation on dilution, as was the case for insulin solutions of the same protein concentration and pH, but higher salt concentration (Gutfreund, 1948*b*).

DISCUSSION

The results of the osmotic pressure measurements recorded in this paper, give further evidence for the hypothesis that insulin molecules in solution consist of aggregates of sub-units of molecular weight 12,000. The mean molecular weight is a function of pH, temperature, protein concentration and ionic strength of the buffer. The reliability of a value obtained for the molecular weight of the sub-unit from the extrapolation, to zero insulin concentra-

tion, of measurements carried out in one buffer system may be subject to suspicion. It has been shown, however, that the graphs representing the dependence of molecular weight upon concentration in a variety of buffers all extrapolate—with different slopes—to the same value of $P/C = 2{\cdot}0 \pm 0{\cdot}1$ at $C = 0$. This agreement is taken as conclusive evidence that the minimum molecular weight of insulin is 11,200–12,300. Mean molecular weight values in heterogeneous solutions obtained from osmotic pressure measurements are always lower than those obtained from kinetic methods, since the former gives a number average which is much more sensitive to the low molecular weight material present than the weight average obtained by the other methods. This sensitivity of osmotic pressure to the presence of small fractions of low molecular weight magnifies the disagreement between the above results and those of Fredericq & Neurath (1950), who state that the molecular weight calculated from the sedimentation and diffusion constants extrapolated to $C = 0$ is 6000. Fredericq & Neurath's experiments were carried out in phosphate buffer pH 2·6, $\mu = 0{\cdot}1$ over a range of protein concentration 1·0–0·2 %. Under those conditions the highly charged insulin molecules will give anomalous values for the sedimentation and diffusion constants, and it is very unlikely that the uncorrected extrapolation is justified. Fredericq & Neurath observed a fall of the sedimentation constant and increase of the diffusion constant on dilution. In our own experiments we found in fact a slight rise in the sedimentation constant with decreasing insulin concentration. This is to be expected from the charge effect. Furthermore, the extrapolation of the diffusion constant is subject to large errors because the method used by Fredericq & Neurath is likely to give progressively low values in very dilute solutions.

Ultracentrifuge runs were carried out as a further test of the hypothesis that the reversible dissociation of insulin is, under the conditions used, limited to division into sub-units of molecular weight 12,000. Though, of course, the molecular weight calculated from the sedimentation constant alone depends upon the hydration and asymmetry of the particles, with the aid of Wyman & Ingalls (1943) nomograms one can easily fix the likely limits for the molecular weight, from the sedimentation constants observed for solutions of insulin in buffer *B*, as 10,000 and 14,000, with $12{,}000 \pm 500$ as the most plausible value. Though there were traces of material of higher molecular weight present, there was no indication of any which sedimented more slowly than the main boundary. These results indicate that the homogeneous sub-unit of insulin has a sedimentation constant $S_{20} = 1{\cdot}65$–$1{\cdot}67 \times 10^{-13}$. Previous experiments at the same pH, but higher salt concentration, showed that the sedimentation constant decreased with decreasing insulin concentration, but it was quite clear from the sedimentation diagrams that the minimum had not been reached (Gutfreund, 1948*b*).

Further evidence about the division of insulin into sub-units of molecular weight 12,000 can be obtained from crystallographic studies. It depends upon the particular crystal class and symmetry whether the unit cell dimensions give values equal to or multiples of the molecular weight. Crowfoot (1938) showed that the unit cell size of the rhombohedral form of insulin, if corrected for 10 % hydration of the air-dried crystal, corresponds to a molecular weight of 36,000. These crystals have the space group R 3, which implies trigonal symmetry. There are, therefore, three identical units within the unit cell, but it is not possible to decide, in this particular case, whether the molecular weight is 36,000 or 12,000. The reasoning developed by Weissenberg (1928) enables one under certain conditions of symmetry to fix the size of the 'crystal molecule' as a fraction of the unit cell. This was possible in the case of orthorhombic insulin crystals grown by Crick & Gutfreund (1950) from acid phosphate buffers. Different crystal forms have been obtained, and one of them is being studied by Mrs D. Hodgkin, F.R.S., and Mr H. Mendel at Oxford. Their preliminary investigations showed that there are four sub-units of molecular weight about 12,000 in the unit cell and the space group is $P2_12_12_1$. Under these conditions the molecular weight is essentially that of the asymmetric unit (12,000), since symmetry conditions show that any bonding which occurs between two sub-units of that size would have to occur between every unit of a chain extending through the macroscopic crystals.

Crystals of insulin sulphate examined by Low (1950) were also orthorhombic and contained four equal units of molecular weight 12,000 in the unit cell. These crystals, however, have the space group $P2_12_12$ which does permit the presence of units of molecular weight 24,000 with a twofold symmetry for each of them.

Chemical evidence does not at present show quite conclusively whether the minimum molecular weight calculated from analytical data is 12,000 or 6000, but it certainly is compatible with the unit of $M = 12{,}000$ (Sanger, 1949) and there is, of course, no reason why this unit should not consist of two identical halves. It is to be hoped that further chemical evidence or physical investigations will show whether the six S—S bridges in the sub-unit are holding the four peptide chains together or whether some of these bridges are used to link a chain with itself, while the pairs of chains are coupled by another kind of bond.

The effect of salt concentration and of the type of ions in the solution upon the aggregation of the sub-unit of insulin can be summarized by the statement that increase in salt concentration causes aggregation, and that there is some indication that sodium chloride has a more marked effect than phosphates. Fredericq & Neurath have shown that potassium thiocyanate favours aggregation even more strongly than sodium chloride. During the last few years it has been shown that a large number of the crystalline proteins have a variable mean molecular weight in solutions. The differences in the conditions of solution, such as salt concentration, pH, protein concentration and temperature, probably cause far more discrepancies in the data obtained than the impurities of the protein preparations to which they are often attributed. Several examples of reversible dissociation into sub-units have been described by Pedersen (1950), Gutfreund (1949) and Tsao, Bailey & Adair (1951). Urea, strong electrolytes and increase in hydrogen-ion concentration cause dissociation of protein into sub-units. High concentrations of strong electrolytes probably act by sheltering the charges on the protein molecules. They cause dissociation in isoelectric haemoglobin or tropomyosin solutions, where the sub-units are held together by opposite charges, while they cause aggregation under conditions when the strong positive charges would otherwise hold the sub-units apart, as described in the above experiments on insulin. Insulin is very stable and such reagents as urea and 0·1 M acid do not cause any irreversible changes since the overall structure is likely to be held so rigidly by the S—S linkages (Sanger, 1949) that a temporary rupture of bonds of electrostatic nature would not cause any change in configuration within the sub-unit. In other proteins the denaturation effect of these reagents is probably due to the slow breaking of hydrogen bonds and a resulting disorder in the protein structure. The large entropy changes of unfolding or stretching chains cause irreversible denaturation and in some cases such a process accompanies the otherwise reversible dissociation into sub-units.

SUMMARY

1. It has been shown that the degree of aggregation of insulin in solutions depends not only upon pH, temperature and insulin concentration but also upon the electrolyte concentration and the type of electrolyte present in the solution.

2. At the optimum pH for dissociation (pH 2·5–2·7) the molecular weight calculated from osmotic pressure measurements extrapolated to zero protein concentration is about 12,000 irrespective of salt concentration. The molecular weight at finite protein concentration is, however, larger the greater the salt concentration. There is some indication that sodium chloride has a more marked effect on the aggregation than phosphates.

3. The sedimentation constant of insulin under conditions of maximum dissociation was found to be $1{\cdot}65 \times 10^{-13}$, which is likely to be the sedimentation constant for the homogeneous unit of $M = 12{,}000$.

4. Some general features of reversible changes occurring in solutions of crystalline proteins are discussed together with structural information which can be adduced from them.

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Some Effects of Cocaine on the Metabolism of *Saccharomyces cerevisiae*

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Since the classical work of Dienert (1900) the phenomenon of enzyme adaptation in micro-organisms has provided material for much research and controversy and several reviews have been written on the subject (Dubos, 1940; Gale, 1943; Spiegelman, 1948, 1950; Hinshelwood, 1950). Enzymic adaptations of a purely quantitative nature have also been demonstrated in mammalian tissues (Cruz, 1946; Davies & Yudkin, 1951).

In recent years much progress in pharmacology and chemotherapy has resulted from the study of the interaction of drugs and enzyme systems. It is possible that the habit-forming properties of certain drugs might be due to their ability to induce adaptive changes in enzyme systems, whereby the cells or tissues become dependent on the presence of the drug for their continued respiratory and metabolic balance. In the present work we have begun our investigations into the validity of this hypothesis by making comparative studies of the effects of habit-forming drugs on the respiration and fermentation of pure strains of yeasts, previously grown in the presence and in the absence of the drug under consideration. We have chosen yeast cells for our preliminary experiments in order to avoid the use of animals for work of an exploratory nature.

In a preliminary report of part of this work (Ryman & Walsh, 1951) evidence was presented that the effect of added vitamin B_1 on the fermentation rate of washed yeast cells was considerably modified, even reversed, with cells which had been grown in the presence of cocaine. The details of these experiments and of investigations into the action of cocaine on the carboxylase activity of *Saccharomyces cerevisiae* are here presented.

MATERIALS AND METHODS

Yeast. Saccharomyces cerevisiae (National Collection of Type Cultures, no. 815) was used throughout this work.

Media. Media having the following composition were employed. (*A*) Malt extract, 20 g., KH_2PO_4, 4 g., K_2HPO_4, 1 g. and glucose 30 g./l., pH 7·0. (*B*) Medium *A* containing 0·004 M-cocaine and the pH adjusted to 7·0. Plugged tubes, each containing 8 ml., were sterilized at 100° for 30 min.

Buffer. Clark and Lub's standard buffer, 0·05 M-phosphate.

Yeast suspension. Tubes of media *A* and *B* respectively, were inoculated with *S. cerevisiae* and incubated at 25°. After a suitable period of growth the cultures were centrifuged, the cells were shaken with buffer, pH 6·0 (5 ml.), and again centrifuged after allowing them to stand for 15 min. This procedure was repeated and followed by a brief washing (approx. 2 min.) with buffer, pH 7·0. The cells from *A* and *B* were suspended finally in equal volumes of distilled water.

Dried yeast suspension. Cells from cultures *A* and *B*, after washing as above, were shaken with acetone (5 ml.), centrifuged at once and dried as rapidly as possible by connecting the centrifuge tubes in parallel to a suction pump.

Sodium pyruvate. Sodium pyruvate was prepared from commercial pyruvic acid by the method of Robertson (1942).

Determination of CO_2. All measurements were made in the Warburg apparatus at 30°. For CO_2 produced at pH 7·0, the contents of the flasks were acidified by the addition of 0·1 ml. 30% (v/v) H_2SO_4 from their side arms, at zero time for the controls and after a suitable interval for the experimental vessels. From the data so obtained, the volumes of CO_2 produced during the experimental period were calculated. The oxygen uptake under the experimental conditions was negligible.

RESULTS

Effects of cocaine on glucose fermentation

The effects of cocaine (0·004 M) on the production of carbon dioxide at pH 7·0 with glucose as substrate by *S. cerevisiae*, previously grown in the presence (*B*) and in the absence (*A*) of cocaine, are compared in Table 1. Higher concentrations of cocaine were found to be unsuitable for these experiments since fermentation rates became somewhat erratic and death of the cells frequently resulted. From preliminary experiments we found that small changes in pH had a considerable effect on the behaviour of the yeast towards cocaine. In each experiment, therefore, the buffer-substrate solution which contained cocaine was carefully adjusted to the same pH as the solution without cocaine.

It can be seen from the table that yeast grown in medium *B* is rather more sensitive towards cocaine than is that grown in medium *A* and that, within the limits of the experiments, this effect is independent of the age of the cultures.

The average ratio of the inhibitions (b/a) is 1·3 (1·2–1·5). In these and in all experiments where the behaviour of yeast grown in the presence of cocaine is compared with that of yeast grown in the absence of cocaine, the suspensions *A* and *B* were freshly and simultaneously prepared from twin cultures. That is, tubes of media *A* and *B* respectively were inoculated at the same time with similar quantities of yeast from the same parent culture. They were incu-

Table 1. *Effect of cocaine on the fermentation of* Saccharomyces cerevisiae

(Each flask contained 3% (w/v) glucose in 0·05 M-phosphate buffer, pH 7·0, with or without 0·006 M-cocaine (2·0 ml.) and yeast suspension (1·0 ml.). For each experiment the yeast suspensions, *A* and *B*, were freshly and simultaneously prepared from twin cultures which had been incubated together. In each experiment suspensions *A* and *B* contained an approximately equal number of cells.)

		A Yeast grown in nutrient medium (no cocaine)			*B* Yeast grown in nutrient medium (0·004 M-cocaine)		
		CO_2 (μl./hr.)			CO_2 (μl./hr.)		
Duration of experiment (min.)	Age of culture (hr.)		Cocaine (0·004 M)	(*a*) Inhibition (%)		Cocaine (0·004 M)	(*b*) Inhibition (%)
45	48	127	97	24	123	80	35
30	48	302	234	23	248	172	31
60	24	209	140	33	146	89	39
60	24	129	97	25	121	82	32
60	48	129	90	30	107	70	35
45	4	143	112	22	153	108	29

Table 2. *Effect of vitamin B_1 on fermentation of glucose by* Saccharomyces cerevisiae

(Each flask contained 3% (w/v) glucose in 0·05 M-phosphate buffer, pH 7·0, ±0·006 M-vitamin B_1 (2·0 ml.) and yeast suspension (1·0 ml.). For each experiment the yeast suspensions, *A* and *B*, were freshly and simultaneously prepared from twin cultures which had been incubated together. In each experiment the suspensions, *A* and *B* contained an approximately equal number of cells. In experiments where the cells were to be in contact with cocaine for several days the twin cultures were subcultured into corresponding media and the subcultures were used.)

			A Yeast grown in nutrient medium (no cocaine)			*B* Yeast grown in nutrient medium (0·004 M-cocaine)		
			CO_2 (μl./hr.)			CO_2 (μl./hr.)		
Duration of experiment (min.)	Age of culture (days)	Total time of *B* on cocaine (days)		Aneurin (0·004 M)	Activation (%)		Aneurin (0·004 M)	Activation (%)
60	2	2	247	274	+11	267	194	−27
60	2	4	202	201	0	206	169	−18
60	2	6	208	218	+ 5	205	176	−14
30	2	4	168	172	+ 2	202	194	− 4
30	2	6	232	248	+ 6	190	164	−14
60	2	3	183	183	0	163	135	−17
60	2	4	111	129	+16	101	96	− 5
60	1	1	140	148	+ 6	162	128	−21
60	2	5	137	140	+ 2	153	139	− 9
60	3	4	150	204	+36	162	158	− 2
60	1	1	178	222	+24	172	163	− 5
60	2	2	129	135	+ 5	129	116	−10

bated and harvested together, and all subsequent procedures were conducted in parallel.

The results of cell counts, made with quantitative dilutions in 1% phenol, indicated that growth occurred at closely similar rates in the twin cultures and, in so far as we were able to judge, the cultures showed no difference in microscopical appearance.

Effects of vitamin B_1 on glucose fermentation

The effects of vitamin B_1 (0·004 M) on the production of carbon dioxide at pH 7·0 with glucose as substrate by *S. cerevisiae* grown in media *A* and *B* are compared in Table 2. The effect in these experiments of the added vitamin B_1 is to stimulate the normal yeast and to depress the cocaine-grown yeast.

Effect of cocaine on carboxylase activity

Carbon dioxide was not produced by the yeast suspension with added pyruvate as substrate. This is in agreement with others who have shown that pyruvate is not decarboxylated by the intact yeast cell (Stoppani, 1951). Dried yeast was used therefore for experiments with pyruvate.

Cocaine (0·004 M) inhibited the carboxylase activity (pH 7·0) of either acetone-dried *S. cere-*

visiae or commercial dried baker's yeast by 80–90%. In a typical experiment with the washed, commercial yeast, inhibitions of 50 and 83% respectively were obtained with 0·002 and 0·004 M cocaine. A concentration of 0·005 M cocaine completely inhibited the carboxylase system. The carboxylase activity was only slightly inhibited by cocaine at pH 6·0.

A comparison of the inhibition by cocaine of carbon dioxide production by yeast grown in medium *A*, with that by yeast grown in medium *B*, was made with washed, acetone-dried *S. cerevisiae*. Pyruvate in optimum concentration, 0·162 M (Green, Herbert & Subrahmanyan, 1941) at pH 7·0 was used as substrate. The effect of cocaine (0·001 M) was to inhibit both the yeast grown in the presence and that grown in the absence of cocaine to a similar extent (45%).

Table 3. *Effect of growth in cocaine on carboxylase activity of* Saccharomyces cerevisiae

(Each flask contained 0·333 M-pyruvate in 0·05 M-phosphate buffer, pH 6·0 (1·0 ml.) and suspension of dried yeast (1·0 ml. ≡ 3–4 mg. dry wt.). For each experiment the yeast suspensions, *A* and *B*, were freshly and simultaneously prepared from twin cultures which had been incubated, harvested and dried together.)

	CO_2 μl./mg. dried yeast/hr.	
Age of culture (days)	*A* Yeast grown in nutrient medium (no cocaine)	*B* Yeast grown in nutrient medium (0·004 M-cocaine)
2	71	74
2	72	73
3	51	48
3	62	67
3	50	46

In view of the high inhibitory effect of cocaine on the carboxylase system, the possibility was investigated that some compensatory adaptation of a quantitative nature might have occurred in the yeast during growth in medium containing cocaine. The carboxylase activity per mg. dry weight of yeast was determined and the results are summarized in Table 3.

DISCUSSION

In spite of the inhibitory effect of cocaine on fermentation, *S. cerevisiae* grows quite well in its presence, provided that the concentration be not greater than 0·004 M (0·005 M inhibits growth completely), and in this respect the organism appears to acquire a tolerance for the drug. Similarly, the yeast which has been grown in the presence of cocaine grows quite well in its absence. On the other hand, the experiments with vitamin B_1 indicate that changes have occurred in the cells as a result of growth in the presence of cocaine and it is quite conceivable that if similar changes occurred in the tissues of a higher animal with a nervous system, they might well contribute towards the establishment of a state of addiction.

The apparent increase in sensitivity of the cocaine-grown yeast towards the drug, as evidenced by the experiments on glucose fermentation, may be a cumulative effect or it may be the result of a change in the permeability of the cell membrane. Danielli (1950) has pointed out that evidence has been accumulating that cocaine exerts its action primarily on the cell surface.

The increase in enzymic toxicity which cocaine exhibits with rise in pH has been observed with other alkaloids; nicotine and morphine, for example, both inhibit cellular respiration at pH 7, but neither exhibit more than negligible activity at pH 6. These observations are in agreement with Rona and others (Rona & Bloch, 1921; Rona & Grassheim, 1923) who concluded from their studies with quinine that the free, un-ionized base was the inhibiting substance. Travell (1940) has demonstrated that the absorption of certain alkaloids including cocaine is dependent on the pH, and she concluded that the rate of absorption is closely related to the concentration of the free, undissociated base. It is interesting to compare the results of these observations with the behaviour of acids, such as hydrazoic and malonic, which inhibit more strongly as the pH falls (Beevers & Simon, 1949).

Cook & McDevitt (1945) have shown that yeast respiration is depressed by cocaine and other local anaesthetics. They observed that this effect was not offset by addition of yeast extracts, but temporary protection was afforded if the extract was added before the drug. These observations are of particular interest in view of our experiments in which we find the fermentation of glucose by cocaine-grown yeast is inhibited by added vitamin B_1, whereas the normal yeast is slightly activated. This effect is small when compared with the results obtained with vitamin B_1 on certain alkali-washed, dried, baker's yeasts in the presence of added cocarboxylase (Ochoa & Peters, 1938; Atkin, Schultz & Frey, 1939; Lipton & Elvehjem, 1940). We have avoided washing with alkali, which dissociates carboxylase (Green *et al.* 1941), in view of the possibility that some modification in the coenzyme might have occurred under the influence of cocaine. The washing procedure adopted was aimed at removing free cocaine.

This modification in the normal function of the vitamin led us to investigate the effects of cocaine on pyruvate metabolism, and it has been shown that the alkaloid is a powerful inhibitor of the carboxylase system. So far, however, we have been unable to demonstrate any direct competition between cocaine and vitamin B_1.

Determinations of the carboxylase activity of the yeast indicate that no quantitative adaptation had occurred as a result of growth in the presence of cocaine.

SUMMARY

1. The effects of cocaine and of added vitamin B_1 on the glucose fermentation of *Saccharomyces cerevisiae* at pH 7, previously grown in the presence and in the absence of cocaine, have been compared. There is a difference in behaviour towards added vitamin B_1 between the normal yeast and the cocaine-grown yeast.

2. Cocaine in concentration sufficient to arrest growth (0·005 M) at pH 7 inhibits completely the carboxylase system of *S. cerevisiae*.

3. The carboxylase systems of yeast grown in the presence and in the absence of cocaine are inhibited by cocaine to a similar extent.

4. No quantitative adaptation of the carboxylase system of *S. cerevisiae* has been found as a result of growth in the presence of cocaine at pH 7.

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Fat Utilization by Muscle

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It has generally been stated that the primary function of the liver in the catabolism of the fatty acids is to convert them into ketone bodies. Only the further oxidation of these intermediate substances was presumed to represent an extra-hepatic function. On the other hand, it has long been questioned whether muscle can also oxidize higher fatty acids directly. Gemmill (1942) found no experimental evidence to indicate the direct utilization of fat by muscle, but this view was not shared by Stadie in his review of this subject in 1945. Cruickshank & Kosterlitz (1941) were able to demonstrate that when cardiac glycogen was low the heart was able to utilize stored fatty acids for its metabolic needs. Lehninger (1946) showed that a rat-heart muscle suspension oxidizes octanoate, laurate and palmitate. Geyer, Matthews & Stare (1949) demonstrated the oxidation of octanoate and trilaurin by various extra-hepatic tissue slices; and Grafflin & Green (1948) had previously shown that kidney enzyme preparations rapidly oxidize all of the saturated monocarboxylic fatty acids. In a recent investigation Goldmann *et al.* (1950) undertook the study of the extent to which the extra-hepatic tissues of the intact animal participate in the oxidation of physiologically occurring fatty acids. Palmitic acid labelled with ^{14}C at the carboxyl position was used, and its conversion to $^{14}CO_2$ was compared in normal and in hepatectomized dogs. Evidence was presented for the view that less than 40 % of the $^{14}CO_2$ exhaled by the normal dog could have resulted from direct oxidation of the labelled palmitic acid by all the extra-hepatic tissues of the animal. At the same time a study of the oxidation of isotopic palmitic acid by various animal tissues was published by Weinhouse, Millington & Volk (1950). Palmitic acid was found to be oxidized *in vitro* by a variety of tissues besides liver; for example, by pigeon-breast muscle, but not by rat skeletal muscle or by rat brain.

In the present study the question of the direct oxidation of fatty acids by muscle *in vitro* is approached from a different angle: we employed Gemmill's diaphragm preparation which makes use of surviving muscle, with serum lipids as substrate, i.e. the natural form of lipids in the living organism.

EXPERIMENTAL

Experimental animals. The experiments were carried out with 3- to 3·5-month-old male rats weighing 90–120 g., from a strain which attains a mature weight of about 250–300 g. in 6–7 months. The animals were maintained under identical conditions of nutrition (wheat and vegetables) and temperature, for at least 1 month before the experiment. The rats were deprived of food for 17 hr. before the experiment. For the experiment the rat was anaesthetized with 1 ml. of 1% pentobarbital, opened and bled by cutting the aorta. The blood was then centrifuged and the serum collected on ice. Another rat, which had been treated identically, was then killed and its diaphragm removed and incubated in the serum of the first rat. By this method the diaphragm was not kept outside the body before incubation any longer than necessary.

Preparation of the diaphragms was carried out according to Gemmill (1940) and Tuerkischer & Wertheimer (1948).

Preparation of the abdominal muscle. In certain cases we replaced the rat diaphragm by the thin abdominal musculature of mice weighing 13–18 g. The abdominal muscle was excised together with the skin which was then peeled off. The muscle was trimmed to give a piece of muscle about 2–3 cm. long and 0·5 cm. wide. This was then cut along the abdominal midline into two nearly equal pieces of tissue. The surrounding medium consisted of the serum of a rat starved overnight before bleeding.

Incubation medium. (1) Serum (1 ml.) was incubated with the diaphragm immediately after centrifugation.

(2) Serum without glucose. About 0·5 g. of freshly washed yeast suspension was added to serum (1 ml.) which was incubated at 37° for 10 min., and subsequently centrifuged. Sugar was removed in this manner in all cases, and then the desired amount of glucose was added. The treatment of serum with yeast had no effect other than that due to removal of glucose. This was shown by the observation that oxidation of fatty acids took place only in the absence of blood glucose and that after the initial removal of blood sugar by yeast, addition of suitable quantities of glucose again abolished fatty acid oxidation.

(3) In order to induce an abnormally high fatty acid content of the serum, we maintained rats on the following diet for 12 days before the experiment: 60% margarine, 20% casein, 14% starch, 3% dried yeast and 3% salt mixture. In addition, the sera of two nephrotic patients with high fatty acid titres were employed.

Method of incubation. The incubation period was 3 hr. The incubation vessels employed were 10 ml. Erlenmeyer flasks. The diaphragms in the medium were gassed for 0·5 min. with O_2 and were then placed in a water bath at 37°, in which the flasks were shaken at the rate of 120 excursions/min.

Chemical procedure. Fat determination was carried out according to the method of Marenzi & Cardini (1943). The lipids are extracted from the blood by Bloor's method. After hydrolysis, the fatty acids are converted into lead salts by addition of lead acetate. The lead salts are converted, by the addition of chromate, into $PbCrO_4$ and this is determined colorimetrically with diphenylcarbazide in an acid medium. In our experience the experimental error of this method was 10%. Employing this method we obtained a mean total fatty acid content of 534 ± 14 mg./100 ml. among 60 rat sera.

Strain and dietary conditions are partially responsible for the high total fatty acid content of our rat sera. We know that the values vary also seasonally. We are working now on this problem.

Measurements of glucose utilization. We used the method described by Krahl & Cori (1947). Glucose was estimated according to Somogyi (1937).

RESULTS

Decrease in fatty acids in glucose-free medium. After incubation for 3 hr., the fatty acid content of the sera had fallen by 23% (Table 1). After 2 hr. incubation the decrease was 21%; while after 5–7 hr. (four experiments) the decrease was 20%. When the simultaneous decreases in fatty acids in the diaphragm and in the surrounding serum were compared, it was found that the fall in titre of the serum

Table 1. *Decrease of fatty acids in serum of varying glucose concentrations, incubated with the diaphragm at 37° for 3 hr.*

No. of experiments	Glucose concn. of serum (mg./100 ml.)	Decrease of serum fatty acids (mg./100 mg. diaphragm)	Decrease of fatty acids in serum (%)
46	0	$1·85 \pm 0·015$	23
5	25	$1·70 \pm 0·03$	18
5	40–50	$1·50 \pm 0·17$	15
14	100	$0·43 \pm 0·19$	6

Table 2. *Decrease of fatty acids in the diaphragm and in serum, after incubation of the diaphragm in glucose-free serum for 3 hr. at 37°*

Exp. no.	Weight of diaphragm (mg.)	Decrease of fatty acids in diaphragm (mg.)	Decrease of fatty acids in serum (mg.)	Decrease of fatty acids Total (mg.)
1	55	+0·430	−0·850	−0·420
2	65	−0·153	−1·40	−1·553
3	63	−0·135	−0·70	−0·835
4	56	−0·440	−0·80	−1·240
5	53	−0·080	−1·10	−1·180
6	81	−0·020	−0·87	−0·890
7	76	−0·180	−0·87	−1·050

was almost always accompanied by a slight decrease in the diaphragm. An absolute total decrease in fatty acids was always obtained (Table 2). When mince of diaphragm or of another muscle was employed, the fall in fatty acids after the same incubation period could not be shown clearly.

Decrease in fatty acids with varying concentrations of blood sugar. In most cases when the serum contained 100 mg. glucose/100 ml., no decrease was observed in serum fatty acids, the mean fatty acid decrease falling within the range of experimental error of the method. With a serum glucose content of 40–50 mg./100 ml. a decrease in fatty acids was demonstrable; and such a decrease was even more marked when the serum glucose content was 25 mg./100 ml. (Table 1). The utilization of sugar by the diaphragm fell with the decrease in the glucose concentration of the serum (Somogyi, 1949). Similar results were obtained when the thin abdominal musculature of the young mouse was substituted for the diaphragm under otherwise identical conditions (Table 3).

Table 3. *Decrease of fatty acids in serum incubated with abdominal muscle of mice at 37° for 3 hr.*

No. of experiments	Glucose concn. of serum (mg./100 ml.)	Decrease of serum fatty acids (mg./100 mg. muscle)	Decrease of fatty acids in serum (%)
7	0	2·0 ±0·10	22
7	100	0·70±0·15	7

Inhibition of fatty acid decrease by metabolites other than glucose. Whereas fatty acid decrease is completely inhibited in the presence of 100 mg. glucose/100 ml. serum, similar quantities of galactose or of acetate were able to inhibit only partially fatty acid utilization. Pyruvate, and in particular acetoacetate, totally inhibited fat utilization (Table 4).

Table 4. *Influence of different metabolites on the decrease of fatty acids in glucose-free serum incubated with the diaphragm for 3 hr. at 37°*

Metabolite added (100 mg./100 ml.)	No. of experiments	Decrease of serum fatty acids (mg./100 mg. diaphragm)	Decrease of fatty acids in serum (%)
Galactose	6	0·79±0·31	12
Fructose	4	0·90±0·33	13
Sodium acetate	8	0·95±0·10	15
Sodium acetoacetate	10	0·33±0·10	5
Sodium pyruvate	6	0·30±0·17	5
Glucose	14	0·43±0·19	6

The effect of metabolic poisons. The addition to the serum of cyanide, fluoride, monoiodoacetate, fluoroacetate or 2:4-dinitrophenol almost entirely inhibited the decrease in fatty acids (Table 5).

Fatty acid decrease following incubation of diaphragm in sera of high fatty acid content. The sera of rats previously maintained on a high fat diet (mean fatty acid content close to 900 mg./100 ml.), and sera of two nephrotic patients (mean fatty acid content of 1425 mg./100 ml.) were used. With the rise in fat content, the decrease in fatty acids after incubation with diaphragm was greatly enhanced, and the inhibitory effect of glucose decreased; so that, even when the blood sugar content was

Table 5. *Influence of several metabolic inhibitors on decrease of fatty acids in glucose-free serum incubated with the diaphragm for 3 hr. at 37°*

Inhibitor	Concn. of inhibitor	No. of experiments	Decrease of serum fatty acids (mg./100 mg. diaphragm)	Decrease of fatty acids in serum (%)
None	—	46	1·85±0·015	23
NaCN	1×10^{-3}–2×10^{-3} M	5	0·60±0·08	5
NaF	2×10^{-3}–$2{\cdot}5\times10^{-2}$ M	7	0·40±0·22	4·6
Monoiodoacetate	5×10^{-3} M	4	0	0
Fluoroacetate	1×10^{-2}–3×10^{-3} M	5	0·50±0·40	4
2:4-Dinitrophenol	1×10^{-4} M	4	0·38±0·17	5

Table 6. *Decrease of fatty acids in sera, of high fatty acid concentration, and various glucose concentrations, after incubation with the diaphragm for 3 hr. at 37°*

No. of experiments	Glucose concn. of sera (mg./100 ml.)	Initial fatty acid concn. (average) (mg./100 ml.)	Decrease of serum fatty acids (mg./100 mg. diaphragm)	Decrease of serum fatty acids (%)
3	50	900*	4·0±0·40	26
6	70		3·0±0·10	31
6	100		0·7±0·26	3
7	0	1424†	9·5±3·9	37
7	70–100		5·5±1·3	30

* Sera of rats which received a fat-rich diet.

† Sera from patients with nephrosis.

normal or super-normal (150 mg./100 ml.), a fatty acid decrease was demonstrable following the customary incubation with diaphragm (Table 6).

DISCUSSION

The absolute decrease of fatty acids in glucose-free serum incubated with rat diaphragm, together with the well-known fact that the R.Q. of the diaphragm of fasting rats when equilibrated without glucose almost invariably yields the theoretical value for fat oxidation (0·7) (Stadie, 1945), leads to the conclusion that the diaphragm is capable of attacking fatty acids directly. Since similar results are obtainable with abdominal muscle of mice, it is permissible to generalize that muscle may be capable of directly breaking down fatty acids. The arrest of fatty acid decrease in the presence of cyanide argues in favour of the assumption that serum fatty acids are oxidized by muscle. Fatty acid decrease is completely inhibited when the normal quantity of blood sugar (100 mg./100 ml.) is present in the serum. In the presence of 40–50 mg. of sugar, fatty acid utilization is demonstrable; and in the presence of 25 mg. it becomes very marked. This fact leads to the problem of the integration of fat and carbohydrate metabolism, in which competition may play an important role. This question has been discussed at some length by Edson (1935) in connexion with studies on hepatic tissue. The supposition is that carbohydrates and fatty acids are in competition for the oxygen available to the normal tissue, and that when both substrates are present carbohydrate is utilized in preference to fatty acids. This idea is not supported by the studies of Weinhouse *et al.* (1949). They compared the rates of oxidation of isotopically labelled short-chain fatty acids in liver slices from previously starved rats with the rates in liver slices of well-fed rats, and they found that oxidation of the fatty acids tended to increase rather than to decrease in the latter case.

According to the results of our study, a competition exists between glucose and fatty acid oxidation in muscle. Pyruvate is also able to inhibit fatty acid oxidation; inhibition can also be shown in the presence of acetoacetate. The possibility exists of an auto-regulation preventing the breakdown of over-large quantities of fat by muscle when a great amount of the ketone bodies is already present.

If, however, an abnormally high quantity of fatty acid is present in the serum, fatty acids are broken down even in the presence of normal or excessive amounts of blood sugar. The amount of fatty acids oxidized under these circumstances is even greater than in the case of normal fat content in the absence of glucose. A rise in fatty acids in the blood may thus be prevented by means of increased breakdown by muscle. Any protracted increase of fatty acids in the blood must therefore be associated either with some disturbance of this breakdown or with an abnormally high mobilization of the fatty acid reserves.

SUMMARY

1. The direct breakdown of the fatty acids of rat serum by resting surviving muscle (diaphragm) has been studied.

2. Fatty acids in the serum decrease when diaphragm is incubated in glucose-free serum for 3 hr. No such decrease is observed when the glucose content of the serum is normal (100 mg./100 ml.). In the presence of 40–50 mg. glucose in 100 ml. serum the decrease is definitely demonstrable; and it is marked in the presence of 25 mg./100 ml.

3. As in the case of glucose, pyruvic acid and particularly acetoacetate completely inhibit fatty acid decrease; whereas inhibition by galactose, fructose and acetate is only partial.

4. Certain metabolic poisons in the customary concentrations completely inhibit the decrease of fatty acids.

5. When diaphragm is incubated in serum of abnormally high fatty acid content and with varying contents of glucose, fatty acid decrease becomes more marked and is less dependent on the glucose content of the serum, so that even with normal glucose content a fall in fatty acids is demonstrable.

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